UNIVERSIDAD AUTÓNOMA DE MADRID

FACULTAD DE CIENCIAS

Departamento de Química Física Aplicada

DIGESTIBILITY OF MILK AND EGG WHITE ALLERGENS. IMMUNOLOGICAL RESPONSES, PEPTIDES RELEASED AND MAPPING OF EPITOPES

DIGESTIBILIDAD DE ALÉRGENOS DE LECHE Y CLARA DE HUEVO. RESPUESTA INMUNOLÓGICA Y CARACTERIZACIÓN DE PÉPTIDOS Y EPÍTOPOS

SARA BENEDÉ PÉREZ

INSTITUTO DE INVESTIGACIÓN EN CIENCIAS DE LA ALIMENTACIÓN (CSIC-UAM)

Madrid 2013

UNIVERSIDAD AUTÓNOMA DE MADRID

FACULTAD DE CIENCIAS

Departamento de Química Física Aplicada

DIGESTIBILIDAD DE ALÉRGENOS DE LECHE Y CLARA DE HUEVO. RESPUESTA INMUNOLÓGICA Y CARACTERIZACIÓN DE PÉPTIDOS Y EPÍTOPOS

Memoria presentada por:

Sara Benedé Pérez

Para optar al grado de

DOCTORA EN CIENCIA Y TECNOLOGÍA DE LOS ALIMENTOS

CON MENCIÓN DE "DOCTORADO INTERNACIONAL"

Instituto de Investigación en Ciencias de la Alimentación

Trabajo realizado bajo la dirección de:

Dra. Elena Molina Hernández

Dr. Iván López Expósito

Dra. Rosina López-Alonso Fandiño

ELENA MOLINA, CIENTÍFICA TITULAR, IVÁN LÓPEZ-EXPÓSITO, INVESTIGADOR CONTRATADO JAE-Doc Y ROSINA LÓPEZ-FANDIÑO, PROFESORA DE INVESTIGACIÓN,

INFORMAN:

Que el trabajo titulado "Digestibility of milk and egg-white allergens. Immunological responses, peptides released and mapping of epitopes / Digestibilidad de alérgenos de leche y clara de huevo. Respuesta inmunológica y caracterización de péptidos y epítopos" constituye la Memoria que presenta Sara Benedé Pérez para optar al grado de Doctora. Esta tesis doctoral se ha realizado bajo su dirección en el Departamento de Bioactividad y Análisis de Alimentos del Instituto de Investigación en Ciencias de la Alimentación (CIAL, CSIC-UAM).

Y para que conste firmamos el presente informe a 4 de octubre de 2013.

Fdo.: Elena Molina Hernández

Fdo.: Iván López-Expósito

Fdo.: Rosina López-Alonso Fandiño

c/ Nicolás Cabrera, 9.
Campus de Cantoblanco
Universidad Autónoma de Madrid
28049 Madrid

A mis padres y a mi abuela

“Las ideas no duran mucho. Hay que hacer algo con ellas.”

Santiago Ramón y Cajal

AGRADECIMIENTOS

Estas líneas, aunque estén al principio de esta tesis, son realmente el punto final de una etapa. Punto al que no habría podido llegar sola y en el cual sólo puedo expresar palabras de agradecimiento para todas las personas que han hecho posible esta aventura.

En primer lugar me gustaría agradecer a mis directores de tesis, los Dres. Elena Molina Hernández, Iván López Expósito y Rosina López Fandiño por permitirme aprender de vuestra experiencia, enseñarme y formar parte de este grupo. Gracias Elena por confiar en mí desde el primer día, por tus consejos y ayuda tanto en lo profesional como en lo personal. Gracias Iván por transmitirme tu experiencia y ser además de un jefe un compañero y gracias Rosina por ser un referente científico a seguir.

Gracias también a las Dras Lourdes Amigo y Mª Victoria Moreno-Arribas, directoras del extinto Instituto de Fermentaciones Industriales (IFI) y del Instituto de Investigación en Ciencias de la Alimentación (CIAL) respectivamente, por poner a mi disposición los recursos técnicos y humanos necesarios para la realización de este trabajo. Y gracias al Consejo Superior de Investigaciones Científicas por la financiación recibida a través de una beca JAE-predoc para la realización de la presente tesis doctoral.

Mi gratitud para las Dras. Margarita Menéndez y Berta Raposo del Instituto de Química Física Rocasolano (IQFR) y del Centro de Biología Molecular Severo Ochoa (CBM) por su ayuda en los experimentos de dicroísmo circular y citometría de flujo respectivamente. Gracias también a las Dras. Marta Reche y Teresa Valbuena del Hospital Infanta Sofia, por la donación del suero de pacientes alérgicos, a las Dras. Inmaculada Pérez y Mª Dolores Ibañez del Hospital Infantil Universitario Niño Jesús, por su colaboración en el trabajo de la lizipaina® y la donación de sueros, y al Dr. Gea del Hospital Universitario La Paz, por la donación de fluidos gastrointestinales.

I had the opportunity to perform two short stays during my PhD. I would like to thank to doctor Clare Mills for the opportunity of taking part of her group at the Institute of Food Research and then at the University of Manchester. I also would like

to thank to the Department of Pediatrics from the Mount Sinai School of Medicine in New York. I am truly thankful to doctors Hugh A. Sampson and Cecilia Berin for allowing me to work in their labs and meet the great people from their groups.

Por supuesto, quiero agradecer a todos y cada uno de los miembros de mi grupo, BIOPEP, su constante ayuda y disponibilidad, especialmente a Alberto Fernández, Beatriz Miralles, Constanza Talavera y Josefina Belloque por su ayuda con la síntesis de péptidos, espectrometría de masas y otras técnicas.

Mención especial merecen todos mis compañeros del IFI y del CIAL, quienes me han alegrado cada día durante la realización de esta tesis. Gracias a los veteranos, Carlos, Marta, Gustavo, Rodrigo, Mª del Mar, Yolanda, David, Inma, Paqui, Wilman, Almu, y especialmente a Dani por sus consejos y ayuda. A los que han entrado después que yo, Laura S., Elvia, Mónica, Sara, Samuel, Alba, Laura P., Silvia, Dani y a aquellos que estuvieron poco tiempo pero que dejaron huella como Marcos, Pedro, Mariana, Nerina, Juli, Victor, Elena, Aurora, Fernanda, Barbara, Blanca, Mafer, Ari…Gracias a todos por los buenos momentos, por vuestra siempre disponible ayuda y por hacer de esta etapa inolvidable.

Por último no puedo dejar de nombrar a mis amigas, gracias por los buenos momentos y vuestros ánimos, y a mi familia, el mayor pilar de mi vida, gracias por estar siempre ahí cuando se os necesita. Especialmente, gracias Alberto por traerme el sol cada mañana, y sobre todo gracias a mis padres por habérmelo dado TODO y apoyarme incondicionalmente, todo lo que he sido, soy y seré es gracias a vosotros.

Sara

CONTENTS

ABSTRACT

Milk and egg allergy is increasing in Europe and the United States, showing a very high incidence, especially in children. Major allergens of these products are glycoproteins, and to induce an allergic response, they should be able to cross the intestinal barrier maintaining sufficient structural integrity to interact with the immune system. Therefore, one of the main characteristics of food allergens is their resistance to gastrointestinal digestion and it is important to assess whether the fragments generated during this process retain IgE binding epitopes. Given the multiple factors involved in physiological digestion, the validity of the stability to the *in vitro* digestion as a criterion to assess the potential allergenicity of proteins is still controversial.

In this thesis, digestions with oral, gastric and duodenal human and simulated fluids of three commercial allergenic food proteins, β-lactoglobulin (β-LG), β-casein (β-CN), and ovalbumin (OVA) have been performed. The results showed that beside the degradation profiles of the two models were different, the residual immunoreactivity, cleavage site of enzymes and the resultant peptides were very similar. The results of microarray and dot blot analysis showed that some of the peptides resulting from digestion maintain their allergenicity being the most immunoreactive areas β-LG (43-65), β-LG (86-99) and β-LG (124-140) in the case of β-LG, β-CN (57-68) and β-CN (82-93) for β-CN and OVA (370-385) for OVA.

Furthermore we also studied the effect of carbohydrate residues attached to the ovomucoid (OM), immunodominant egg allergen, on its allergenicity and digestibility. Results indicated that after enzymatic deglycosylation, the structure of OM was unmodified and 80% of the patients used in the study showed reduced IgE binding capacity compared with OM glycosylated. The presence of IgE-reactive epitopes specific for glycosylated protein was also demonstrated. Deglycosylated OM was more

susceptible to proteolysis during digestion but the resultant peptide pattern was very similar. Immunoreactivity of the digests measured by inhibition ELISA decreased to values between 1 and 4% at the end of digestion, but some of the identified peptides such as OM (80-89) and the regions OM (36-61), OM (100-122) and OM (133-180) showed maintain their IgE binding capacity reacting in 40-100% of patients tested.

The last part of this thesis describes the identification of peptides generated during the *in vitro* gastrointestinal digestion of a major egg allergen, lysozyme (LYS). The results indicated that the gastric and duodenal digests retained their IgE binding and basophils activation capacities. This biological activity may be attributed to the presence of intact LYS, to the fragment LYS (24-129) produced by the action of chymotrypsin or to the immunoreactive peptides released during digestion and linked by disulphide bridges, containing the epitopes LYS (57-83) and LYS (108-122).

The fact that major linear epitopes can be released during digestion of LYS led to study the case of an egg allergic child, which tolerate egg after a immunotherapy treatment, suffered a severe allergic reaction to a medication containing LYS and papain The hypothesis that the presence of papain, could increase the allergenic potential of the LYS was raised. The results indicated that LYS treated with papain produced fragments with a high IgE-binding capacity and they were identified as LYS (22-129), LYS (34-96) and LYS (62-128). Furthermore, these fragments could be linked by disulphide bridges forming large structures that could have an IgE-binding capacity increased.

RESUMEN

La alergia a leche y huevo presenta una alta incidencia y va en aumento en Europa y Estados Unidos, sobre todo en la población infantil. Los principales alérgenos de estos productos son glicoproteínas, y para inducir una respuesta alérgica, deben ser capaces de atravesar la barrera intestinal manteniendo la suficiente integridad estructural como para interactuar con el sistema inmune. Por ello, una de las características principales de los alérgenos alimentarios es su resistencia a la digestión gastrointestinal y es importante evaluar si los fragmentos generados durante este proceso retienen epítopos de unión a IgE. Dados los múltiples factores que intervienen en la digestión fisiológica, la validez de la estabilidad frente a la digestión *in vitro* como criterio para estimar el potencial alergénico de las proteínas está siendo muy discutida.

Durante la realización de la tesis doctoral, se han llevado a cabo digestiones con fluidos orales, gástricos y duodenales humanos y simulados con enzimas comerciales de tres proteínas alimentarias alergénicas, β-Lactoglobulina (β-LG), β-caseína β-CN, y Ovoalbumina (OVA). Los resultados mostraron que aunque los perfiles de degradación de los dos modelos fueron diferentes, la inmunoreactividad residual de los digeridos, los puntos de corte de las enzimas y los péptidos resultantes fueron muy similares. Los resultados de los análisis por microarray y dot blot mostraron que aLGunos de los péptidos resultantes de la digestión mantienen su capacidad alergénica. Siendo las áreas inmunorreactivas más destacadas, β-LG (43-65), β-LG (86-99) y β-LG (124-140) en el caso de la β-LG, β-CN (57-68) y β-CN (82-93) para β-CN y OVA (370-385) para OVA.

Por otro lado también se estudió el efecto de los residuos de carbohidratos unidos al ovomucoide (OM), alérgeno inmunodominante del huevo, en su alergenicidad y digestibilidad. Se comprobó que tras la desglicosilación enzimática del OM, la proteína mantenía su estructura inalterada y 80% de los pacientes estudiados mostraron una menor

capacidad de unión a IgE comparado con el OM glicosilado. Además, se demostró la presencia de epítopos reactivos frente a IgE específicos para la proteína glicosilada. El OM desglicosilado fue más susceptible a la proteólisis durante la digestión pero el patrón peptídico resultante fue muy parecido. La inmunorreactividad de los digeridos medida por ELISA de inhibición disminuyó hasta valores de entre el 1 y el 4% al final de la digestión pero algunos de los péptidos identificados demostraron mantener su capacidad de unión a IgE entre los que destacan el péptido OM (80-89) y las regiones OM (36-61), OM (100-122) y OM (133-180) que reaccionaron en el 40-100% de los pacientes ensayados.

La última parte de la tesis doctoral ha consistido en la identificación de los péptidos generados durante la digestión gastrointestinal *in vitro* de uno de los mayores alérgenos del huevo, la lisozima (LYS). Los resultados indicaron que los digeridos gástrico y duodenal mantenían su capacidad de unión a IgE y de activar basófilos. Esta actividad biológica podría ser atribuida a la presencia de LYS intacta, al fragmento (24-129) producido por la acción de la quimiotripsina o a la liberación durante la digestión de péptidos inmunorreactivos unidos por puentes disulfuro que contenían los epítopos LYS (57-83) y LYS (108-122).

El hecho de que importantes epítopos lineales puedan ser liberados durante la digestión de la LYS llevó a estudiar el caso de un niño alérgico al huevo, que tolerando huevo después de un tratamiento de inmunoterapia, sufrió una fuerte reacción alérgica a un medicamento que contenía LYS y papaína por lo que se planteó la hipótesis de que la presencia de papaína, podría incrementar el potencial alergénico de la LYS. Los resultados indicaron que la LYS tratada con papaína dio lugar a fragmentos con una alta capacidad de unión a IgE que fueron identificados como LYS (22-129), LYS (34-96) y LYS (62-128). Además estos fragmentos podrían permanecer unidos por puentes

disulfuro formando grandes estructuras que podrían tener una capacidad de unión a IgE aumentada.

ABBREVIATIONS

APC: Antigen presenting cells

BSA: Bovine serum albumin

CN: Casein

Da: Daltons

dOM: deglycosylated ovomucoid

ELISA: Enzyme Linked ImmunoSorbent Assay

HSA: Human serum albumin

IC50: Sample concentration which reduces the IgE binding to 50%

IgE: Immunoglobulin E

LF: Lactoferrin

LYS: Lysozyme

MALDI: Matrix-assisted laser desorption/ionization

MS/MS: Tandem mass spectrometry

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide

OM: Ovomucoid

OVA: Ovalbumin

PBMCs: Peripheral blood mononuclear cells

PBS: Phosphate buffer solution

PBS: Phosphate buffer solution containing Tween 20

PC: Phosphatidylcholine

ppm: parts per milliion

RP-HPLC: Reverse phase-High performance liquid cromatography

SDS-PAGE:Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TBS: Tris buffer solution

TBS-T: Tris buffer solution containing Tween 20

TOF: Time of flight

α-LA: α-Lactalbumin

β-LG: β-Lactoglobulin

1. INTRODUCTION

1.1. FOOD ALLERGY OVERVIEW

The newly created International collaboration in Asthma, Allergy and Immunology (iCAALL) defines food allergy as an "adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food" (Boyce et al., 2010). This definition agrees with other international guidelines and encompasses immune responses that are IgE-mediated, non-IgE-mediated, or a combination of both (Sackeyfio et al., 2011; Urisu et al., 2011). IgE-mediated food allergy is believed to be responsible for most of the food induced hypersensitivity reactions and it is characterized by an acute onset of symptoms usually within 2 hours after ingestion or exposure to a food product. Those symptoms typically involve the skin (urticaria and angioedema), gastrointestinal tract (vomiting, diarrhea, abdominal pain), respiratory tract (asthma and rhinitis) and, in the most severe cases, they may result in a rapid and progressive systemic reaction involving multiple systems that might end up in a cardiovascular collapse (anaphylaxis) (Burks et al., 2012). Unfortunately, the current management of food allergy is limited to strict dietary avoidance, nutritional counselling and emergency treatment of adverse reactions. There have been attempts to desensitize patients with food allergy for more than 100 years (Schofield, 1908), however there are still no accepted therapies to accelerate the development of oral tolerance or to provide effective protection from unintentional exposures (Nowak-Wegzryn & Sampson, 2011).

Briefly, allergic sensitization occurs when the allergen enters the body, typically through the mucous membranes, and it is taken by antigen presenting cells, which eventually trigger the differentiation of naïve allergen-specific T cells into Th2 cells. This is followed by the activation of B lymphocytes into IgE antibody-producing plasma cells. The specific-IgE antibodies bind to the surface of tissue mast cells and blood basophils so

that, on re-exposure to the food, antigenic proteins bind to and cross-link these cell surface-bound specific IgE antibodies, triggering the release of symptom-causing mediators, such as histamine and leukotrienes (Figure 1) (López-Expósito et al., 2013). Subjects can be allergically sensitized and produce specific-IgE to food allergens without having clinical symptoms of an allergic reaction on exposure. Thus, sensitization alone is not sufficient to define food allergy. There have to be specific signs and symptoms on exposure to the offending food together with a measurable food-specific IgE (Boyce et al., 2010).

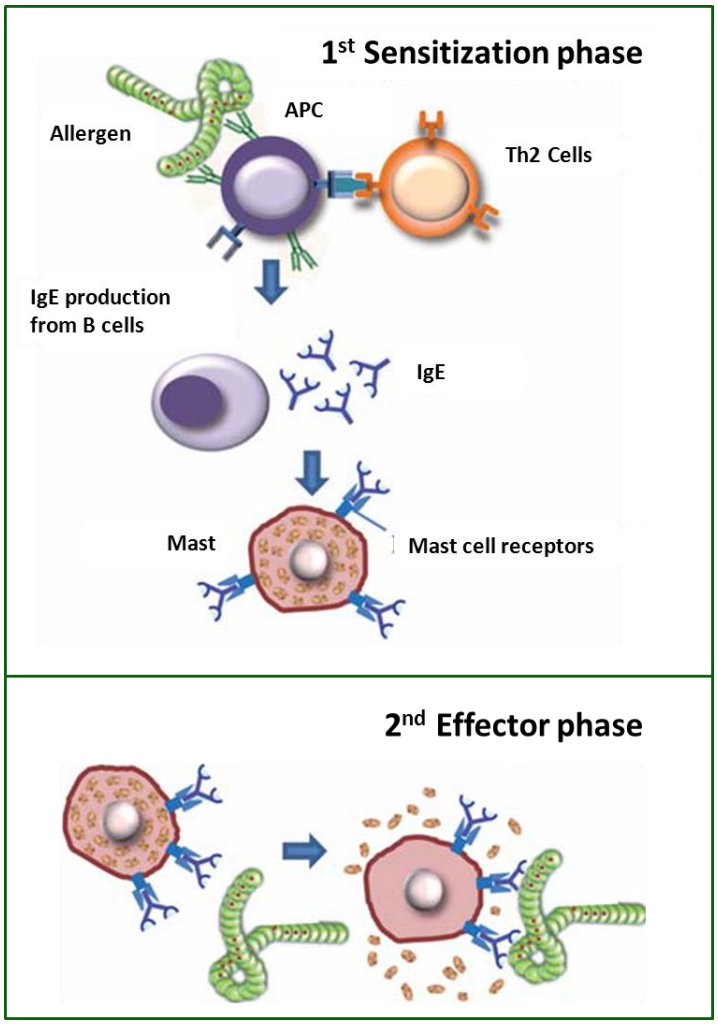

Figure 1. Schematic representation of the events leading to sensitization and allergic response. (López-Expósito et al., 2013).

Nowadays the causes responsible for food allergies remain unknown, although it has been recognized that several environmental and mainly genetic factors might influence susceptibility to food allergy. The true prevalence of food allergy is difficult to establish because most prevalence studies differ in their design and in the definition of food allergy, or focus only on the most common foods (Boyce et al., 2010). The World Allergy Organization estimated the frequency of food allergy in 8% of children and 2% of adults (Sicherer et al., 2004; Pereira et al., 2005; Venter et al., 2008). However, there are wide variations in each country with respect to the most common food allergies, which suggests that, in addition to a significant genetic component to food allergy, there is also a significant contribution of local diet (Branum and Lukacs, 2009; Sicherer, 2011) as well as of other factors, such as the timing of first exposure, the performance of the digestive processes or the diversity of gut microbiota, many of which have not yet been identified (Berin, 2013).

More than 170 foods are reported to cause food allergies, but only eight "the big 8" (peanut, tree nuts, milk, egg, wheat, soy, fish and shellfish) account for 90% of all food-allergic reactions (Boyce et al., 2010), being egg and milk the most common offending foods in children from continental Europe (Rona et al., 2007). A meta-analysis involving double-blind placebo-controlled food challenges confirmed a varied prevalence from 0.2% to 7% of egg allergy and from 1.2% to 17% of milk allergy (Rona et al., 2007). Egg and milk allergy mainly affect children below the age of three, and although most of them outgrow their allergy by the age of 16 years, a significant proportion of the population retains the allergy throughout life (Savage et al., 2007; Skaripak et al., 2007).

Allergic reactions to egg and milk occur, sometimes, after the first known exposure to the food, therefore it has been speculated that they could be attributed to

uterine sensitization (Vance et al., 2005) or contact through breast milk (Denis et al., 2012) but this theory is controversial. Other possible routes of sensitization are respiratory, due to inhalation of egg and milk particles in dust (Roberts and Lack, 2003), and cutaneous, described as bird-egg syndrome in some patients sensitized to egg through avian proteins, as alpha-livetin (Gal d 5), by contact with feathers or bird droppings (Nowak-Wegrzyn and Sampson, 2006). There are also cross-reactions between egg and chicken meat, among the proteins from egg white and yolk (Besler et al., 2001) and between eggs from different birds (Fox and Lack, 2003), as well as between milk from different species (Restani et al., 1999). However, the most common route of sensitization is through the gastrointestinal tract (Lehrer et al., 1996; Mills et al., 2003; Bannon, 2004).

1.2. DIGESTION AND FOOD ALLERGY

❖ *1.2.1. Digestibility of food allergens*

Most relevant allergens are glycoproteins, soluble in water, with a molecular mass between 10 and 70 kDa, and stable to heat, acid and proteases. Only some regions of the proteins are recognized by the immune system. Such regions are called epitopes and can be linear (uninterrupted sequences of amino acids) or conformational (maintained due to the tertiary structure). Because of their stability against acids and proteases, digestive functionality and gut permeability are considerer key factors that may affect the allergenic potential (Lehrer et al., 1996) (Moreno, 2007). Moreover, although there are no molecular properties common to food allergens, stability to digestion is a shared feature among the allergenic food proteins (Metcalfe et al., 1996) (Taylor and Leher, 1996). The abundance in the food and the ability to develop a biological function and to maintain a stable

structure influence digestibility, bioavailability and the subsequent allergenicity of a protein.

Most allergens such as casein (CN) from milk or ovalbumin from egg, represent between **20 to 80% of the total protein content of the food.** Therefore it appears that allergenicity is related to the degree of exposure throught the gastrointestinal tract to a particular protein. However, it is remarkable that there are abundant proteins that are not allergens, indicating that the intrinsic immunogenicity of a protein is more important than the exposure dose (Taylor and Lehrer, 1996).

It is difficult to establish a relationship between **biological function** and allergenicity but many allergenic proteins have a particular biological activity. Enzymes such as papain, with cysteine protease activity (Baur et al., 1982), or lysozyme with muramidase activity (Holen and Elsayed, 1996), are good examples, because they are relatively stable in hostile environments in order to carry out their duties efficiently. Other allergenic proteins exert transport, storage and ligand-binding functions. This is the case of β-LG, whose primary function appears to be the transport of retinol (Ball et al. 1994), which reduces the mobility of the protein peptide chain and gives rise to an increased resistance to heat treatment and digestion (Douliez et al., 2001).

The mechanisms which confer a high integrity to **protein structure** may be important in the stability during digestion. Ovotransferrin and ovomucoid with fifteen and nine disulphide bridges respectively, or β-LG with a great tendency to adopt beta structures (Thompson and Eisenberg, 1999) are good examples of this. Other allergens such as CNs, unlike globular proteins which possess well-defined three-dimensional structures, contain large regions of disordered structure (Dunker et al., 2001). These

proteins, called reomorphic, not undergo an abrupt transition from one conformational state to another, but they adopt a dynamic set of secondary structures which are in equilibrium with each other and preserve stable potential epitopes (Holt and Sawyer, 1993). Glycosylation can have a significant stabilizing effect on protein structure modifying the physical properties of the protein, altering its solubility, hydrophobicity and electric charge, among others, affecting their stability (Wormald, 1991) and exerting a protective effect against gastroduodenal digestion. It has been shown that N-glycosylation can have a significant stabilizing effect on protein structure (wormald and Dwek, 1999) and there is evidence that it increases the stability of, for example, the 7S globulin of pea and its resistance to chemical denaturation (Pedrosa et al., 2000). Moreover, covalent modification of the proteins through Maillard reactions resulting in products that have been shown to destabilize the quaternary structure of proteins, such as reducing the stability at low pH of the triple helix of collagen (Breiteneder and Mills, 2005).

Other physicochemical features of the proteins could also influence their stability during digestion. The trend of some proteins to aggregate under physiological conditions, or as result of food processing, may increase their allergenicity (Chirino et al., 2004), probably due to the presentation of multiple IgE epitopes, which may lead to histamine release with greater efficiency than soluble monomeric forms. In fact, it has been indicated that the aggregates with a molecular weight greater than 100 kDa are efficient inductors of the immune response (Rosenberg, 2006). Jiménez-Saiz et al. (2011b) found that lysozyme could aggregate and precipitate at pH values and surfactant concentrations typical of the duodenal medium helping it to resist digestion by pancreatic enzymes.

❖ 1.2.2. Food processing

Food processing can induce physical and chemical modifications to the proteins, such as aggregation or denaturation among others, affecting their structure and their resistance to digestive enzymes. For instance, it has been shown that β-LG resistance to digestive enzymes is reduced under heat treatment in a temperature-dependent manner (Ehn et al., 2004; Taheri-Kafrani et al., 2009; Morisawa et al., 2009). During processing, proteins can also interact with other components of the food matrix and consequently their allergenic properties, their digestibility or both can be modified (Teuber, 2002). Examples include protein- sugar interactions that occur during heating such as Maillard reaction (Gruber et al., 2004), or interactions with food lipids forming emulsions (Breiteneder and Mills, 2005). Corzo-Martínez et al. (2010) demonstrated that interaction with sugars induced by Maillard reaction protect β-LG during *in vitro* gastrointestinal digestion. On the other hand, Jiménez-Saiz et al. (2012) showed that the presence of egg proteins as part of an emulsion do not change their IgE binding, but it slightly increase the digestibility of the main allergens present in the egg-white. It has also been reported that the polymerization of chemically denatured β-LG facilitate the action of the gastrointestinal enzymes, and their digestion products present low antigenic properties (Villas-Boas et al., 2012).

❖ 1.2.3. Digestion parameters

Proteins with the capacity to induce sensitization and elicitation of an allergic response must retain, after digestion, sufficient structural integrity to induce T-cell differentiation and IgE-mediated activation of effector cells (Polovic et al., 2007; Ruiter and Shreffler, 2012). In this regard, the degree of hydrolysis achieved during digestion,

determined to a great extent by the conditions of the digestive tract, defines the peptide fragments that interact with the intestinal mucosa where absorption occurs and their IgE binding and T-cell stimulating capacities. In this respect, when using *in vitro* models to simulate digestion, the choice of proteolytic enzymes, digestion conditions, and methods for the analysis of protein hydrolysates have a considerable impact on protein digestibility assessment.

1.2.3.1. Enzymes

Different enzymes with different functions are produced along the digestive tract but the most relevant to protein digestion studies are, without any doubt, proteinases and peptidases such us pepsin, trypsin, chymotrypsin and carboxypeptidases. It is difficult to know the amount of enzymes secreted under physiological conditions because there is a wide individual variation, and therefore it is not easy to choose an optimal enzyme to substrate ratio. It has been estimated that pepsin secretion in adults is between 20 and 30 kUnits of enzyme activity/24 h at 37 °C whereas a typical adult dietary intake of protein could be estimated around 75 g/24 h (Moreno et al., 2005). These estimations clearly indicate that protein would normally exceed pepsin but it has been stated that pepsin in human gastric samples can vary over 10,000 fold (Moreno et al., 2007) and depending on the amounts used, the degradation of the protein will occur with to various extents (Fu et al., 2002). Due to the difficulty in obtaining human enzymes, bovine and porcine enzymes are mainly used. Thus, it is important to considerer differences in the specificity of the enzymes to be used in experimental models (Guillou et al., 1991).

1.2.3.2. pH

The pH is critical in gastrointestinal hydrolysis of proteins because it directly affects the degree of activity of the digestive enzymes. Pepsin activity is optimal at pHs

between 1.8 and 3.2 (Jensen-Jarolim, 2006) with any elevation in the pH having a negative effect on peptic degradation (Untersmayr and Jensen-Jarolim, 2006). The gastric pH can oscillate substantially depending on the age, (Koldovski, 1984) pre- or postprandial conditions or ingested food (Tyssandier et al., 2003). Adult stomach under fasting conditions presents a pH of about 2, but this value may increase to more than 4 after the food intake (Tyssandier et al., 2003). In addition, the acidity of the stomach in children is much lower, with a pH value of about 4 (Schmidt et al., 1995). Extrinsic factors such as the consumption of antacids in patients in which the digestive process is compromised can enhance the pH found in the stomach increasing risk of sensitization to certain food allergen (Untersmayr et al., 2008). The pH of the intestine is also important because, it can also influence the activity of some enzymes (Hamosh et al., 1981), and although it has been described that may vary within the range 5-7 (Friend, 1991). The pH also modified protein structure and flexibility (Horng et al., 2003) and therefore, the digestibility pattern of the allergens (Moreno et al., 2005).

1.2.3.3. Bile salts

Bile is an alkaline solution consisting of water, hormones, pigments, electrolytes (Na^+, K^+, Ca^+, Cl^-, HCO_3^-) and lipids. Among lipids, the most important according to their abundance are bile salts (6-10%), phospholipids (95% phosphatidylcholine, PC) and cholesterol (0.5-5%). In the duodenal environment, these three lipids interact hydrophobically to form mixed micelles and liposomes, but phospholipids such as PC, is also secreted by the gastric mucosa, so are present in the stomach at an early stage of the digestion process. It has been shown that interactions between PC and proteins can impact on rates of digestion protecting or making the protein more susceptible against digestion. In this regard, it was shown that the low pH found in the stomach (pH 2.5) induced a

structural change in the α-LA to the molten globule state enabling it to penetrate the PC vesicles and this was thought to be the mechanism responsible for decreasing the rate of simulated gastric pepsinolysis (Moreno et al., 2005). In contrast, bile salts could bind into the hydrophobic pockets of the β-LG, which might destabilise the protein structure and made additional interior domains of the protein available for the protease action (Gass et al., 2007).

1.2.3.4. Other factors

Others factors, such as, gastric emptying time (McClements et al., 2009), ionic strength of the digestive fluids (Boisen and Eggum, 1991), mechanical stresses (Minekus et al., 1995) or substrate concentration (Boisen and Eggum, 1991) could also play an important role in the digestibility of proteins as well as the maturity of the gut and its healthy functioning. In this regard, it is important to take into account the conditions found in the immature gastrointestinal tract of infants. A model of infant gastrointestinal digestion, with pH between 3 and 4, an enzymes to substrates ratios lower than in the adult model and with reduced concentrations of PC and bile salts, should be used when the objective of the study is focus on infant digestion instead of using general conditions of digestion which reflect the mature adult digestive system.

❖ *1.2.4. Food digestion models*

In vivo research on food digestion is ethically and technically difficult and also expensive to perform. Therefore, ***in vitro*** digestion models have been developed over the years. Some models focus on one particular region of the gastrointestinal tract (Fu et al., 2002; Jakobsson et al., 1982; Chatterton et al., 2004), whereas other models are

developed following a number of sequential steps (Moreno et al., 2005), usually gastric and duodenal. Moreover there are discrepancies between models in the kind and amount of enzymes used. One of the most broadly accepted models was described by Moreno et al. (2005) who used physiologically relevant conditions of digestion based on *in vivo* data obtained from gastric and duodenal aspirations. In this line there is an action granted by the European Cooperation in Science and Technology (COST) within the domain of food and agriculture, "Infogest", which is trying to harmonise *in vitro* digestion methods in a unique static *in vitro* digestion model validated with available *in vivo* data.

If the samples are solid the mechanical effect of the mouth could be crucial. Similarly, engineered *in vitro* dynamic models have been developed to mimic stomach contractions (Kong and Singh, 2010), peristaltic movements and intestinal flow (Spratt et al., 2005). In this regard, a complete artificial digestive system of the human upper gastrointestinal, including a food matrix as part of the design, has been patented by Mainville et al. (2005). In any case, and in order to mimic the physiological conditions of digestion, the characterization of human gastroduodenal juices (Ulleberg et al., 2011) is essential to develop an *in vitro* digestion model as close as possible to the *in vivo* conditions. In fact, results so far have demonstrated that proteins digested by human gastroduodenal juices produced different peptides as compared to those digesed with porcine digestive enzymes (Eriksen et al., 2010) highlighting the importance of a comparison between studies.

The logical step after performing *in vitro* digestion models is to validate them against data collected ***in vivo***. There are some digestion studies in humans collecting the gastric and duodenal effluents after ingestion of food (Mahé et al., 1991; Mahé et al., 1996; Kuwata et al., 1998; Furlund et al., 2012), but the results so far are controversial.

Because of the difficulty and expense associated with human fluids, several animal models such as piglets (Bouzerzour et al., 2012), calves (Scanff et al., 1992), mice (Joo and Kato, 2006) or rats (Kitabatake and Kinekawa, 1998) are an alternative to human digestibility studies.

Another approach to the study of digestibility is based on in silico methods (Pal et al 2004; Ferrua and Singh, 2010). Jiang et al. (2007) performed a computational analysis of the relationship between allergenicity and digestibility. Food allergen data were obtained from a structural database of allergenic proteins and were subjected to a virtual digestion using two proteolytic enzymes present in human stomach during the digestion. They concluded that according to their *in silico* resistance to digestion, the allergens could be classified into two groups according their pathway for sensitization, gastrointestinal tract sensitizing and non-gastrointestinal tract sensitizing allergens, being the former relatively resistant to gastric fluid digestion while the later are relatively labile. A recent study, tried to found descriptors of intestinal stability. For that, 228 dipeptides were synthesized and their intestinal stability was obtained by *in vitro* digestion. Database were constructed to study the relationship between structure and stability showing the N-terminal residues Asp, Gly, and Pro as well as the C-terminal Pro, Ser, Thr, and Asp as peptide stabilizing factors (Foltz et al., 2009). Computational simulation has the potential to substitute manual experiments but despite the advances made to date, more research and validation with real data are needed to develop an accurate *in silico* digestion model.

1.3. EFFECT OF GASTROINTESTINAL DIGESTION ON THE ALLERGENICITY OF MILK AND EGG PROTEINS

To date, there are several studies about digestibility of allergenic proteins, but establishing a comparison of the results is difficult because most of them employ different models of digestion with different enzymes, conditions and enzyme to substrate relationships. In addition, only a few of the published studies try to find a correlation between the hydrolysis products and the allergic response. This section of the introduction will review the main research carried out regarding the digestibility of milk and egg allergens. Results regarding protein degradation, models employed and immunoreactivity of the resultant products will be discussed.

❖ *1.3.1. Milk proteins*

Cow's milk contains between 3-3.5% proteins and includes approximately 20 different proteins that may be involved in allergic sensitization (Herz, 2008). Milk proteins are classified as caseins (CNs, Bos d8), which constitute 80% of the total milk proteins and they are described as the most allergenic ones, and whey proteins (Monaci et al., 2006), being β-lactoglobulin (Bos d 5) and α-lactalbumin (Bos d 4) the major allergens from this fraction (Table 1).

Table 1. Main proteins in bovine milk.

Milk Proteins	% of total protein content	Concentration in milk (g/L)
Whey proteins	20	
β-lactoglobulin	10	3-4
α-lactalbumin	5	1-1.5
Immunoglobulin	3	0.6-1
Bovine serum albumin	1	0.1-0.4
Lactoferrin	Traces	0.09
Whole Casein	80	
α_{S1}-Casein	32	12-15
α_{S2}-Casein	10	3-4
β-Casein	28	9-11
κ-Casein	10	3-4

1.3.1.1. Caseins

CNs are considered the most allergenic proteins in milk, as it was demonstrated by Shek et al. (2005) who reported that patients with IgE-mediated cow´s milk allergy had higher specific IgE concentrations to casein compared with whey proteins. Because of that, a number of studies regarding the digestibility and allergenicity of the different casein types (β-, κ- and α_s-CN) have been carried out. Resistance to pepsinolysis of β-CN, one of the most immunoreactive fractions, was tested using different enzymatic sources and different pHs, such as porcine pepsin at pHs ranging from 1.2 to 3.0 (Mandalari et al., 2009a; Dupont et al., 2010a), and human gastric fluid at pHs from 2 to 6.5 (Jakobsson et al., 1982; Chatterton et al., 2004). All those experiments agreed to conclude that β-CN was easily degraded by pepsin. Some differences were detected by Dupont et al. (2010a) when they compared an infant gastric digestion model at pH 3, with an adult model at pH 2.5. They observed that bovine β-CN was digested more slowly in the infant model, not

only due to the higher pH but also to a 8-fold reduction in pepsin concentration. Furthermore, changes in the hydrophobicity of β-CN at pH 3.0 might contribute to its resistance to pepsinolysis. Using human gastric fluid, Chatterton et al. (2004) observed that bovine β-CN, as part of a whey protein concentrate, and human β-CN were completely degraded by human gastric juice at pH levels lower than 4. This fact is remarkable because gastric pH is not expected to fall below 4.0 in healthy term infants (Mason, 1962). However, α_{s1}-CN was particularly resistant to the effects of gastric proteases even at pH 2.0. On the contrary, Fu et al. (2002) reported that α_s-CN was completely degraded after 1 min of porcine pepsin digestion, but probably because of a higher ratio of pepsin to protein was used, which was about 13:1 (w:w), and to the pH 1.2. *In vivo* studies have been reported by Mahé et al. (1991) showing the presence of intact CN in gastric aspirates collected 1 hour after drinking skim milk, probably because of the high pH measured of the effluents, with values between 4 and 6 during the digestion process.

A crucial information to take into account when evaluating the digestibility and allergenicity of food proteins is the matrix effect. Macierzanka et al. (2009) compared β-CN gastric digestion when the protein was presented in solution or as part of an emulsion. The authors found that digestion of β-CN was faster when presented as an emulsion, suggesting that there were changes in the adsorved β-CN conformation that allowed pepsin to cleave the protein more effectively than in solution. Moreover, when they studied the effect of the inclusion of PC in the digestion model, they observed that when the protein was in solution, the kinetics of pepsinolysis was not affected, but when β-CN was in an olive oil-in-water emulsion the digestion rate was reduced because PC displaced the protein from the oil droplet surface so that gastric digestion mainly take

place in solution. Similarly, Dupont et al. (2010a) did not report a significant effect of PC on gastric hydrolysis of β-CN in solution.

CNs are also susceptible to hydrolysis by duodenal enzymes as it was demonstrated by Mandalari et al. (2009a) and Jakobson et al. (1982) when β-CN was directly digested with pancreatin and human duodenal fluid respectively, without a previous gastric stage. α_s-CN was also easily digested by duodenal enzymes (Fu et al., 2002). There are a few studies dealing with gastric digestion of β-CN followed by a duodenal stage (Macierzanka et al., 2009; Mandalari et al., 2009a; Dupont et al., 2010a; Dupont et al., 2010b; Alessandri et al., 2012). As expected, intact β-CN was not found at the end of the complete gastrointestinal digestion despite the use of different matrixes, pHs and enzyme concentrations in the different studies. Similar results were reported for κ-CN (Dupont et al., 2010b), α_s-CN (Morisawa et al., 2009) and β-CN with the use of human gastric and duodenal fluids (Jakobsson et al., 1982). Although the previously described studies have investigated different aspects of β-CN digestion, the residual IgE-binding or the presence of IgE epitopes in the digestion products has not been evaluated.

1.3.1.2. β-lactoglobulin

Most of the available *in vitro* studies on digestibility of bovine β-LG use porcine pepsin in the simulated gastric phase, but they differ in the pepsin:β-LG ratio used. However, results agree that native bovine β-LG is highly resistant to peptidic digestion, although they differ on the percentage of intact protein remaining after pepsinolysis with values from 68% (Bossios et al., 2011) to 100% (Mandalari et al., 2009a), possibly because between both studies there is a difference in the concentration of pepsin of 19-

fold. This resistance is probably due to its special folding forming a β-barrel core structure where the cleavage sites of pepsin could be located (Dalgalarrondo et al., 1995). In addition, variant B has been shown to be more resistant than A against pepsinolysis (Fu et al., 2002). Pepsinolysis resistance is also dependent of the β-LG species as it has been demonstrated that caprine β-LG is easily digested than bovine B-LG (Almaas et al., 2006a).

It has also been demonstrated that bovine β-LG could not be hydrolysed by human pepsin when human gastric fluid was used (Chatterton et al., 2004; Jakobsson et al. 1982; Almaas et al., 2006b) but this fact was problably because they used a low amount of fluid to performed the digestion of a high concentration of protein and therefore the enzyme to substrate ratio employed was very low.

β-LG has also shown to be resistant to human pepsin when it is involved in skim cow milk (Almaas et al., 2006a), whey protein concentrate (Chatterton et al. 2004) or phosphate buffer (Jakobsson et al. 1982) and to porcine pepsin in whey protein isolated (Kitabatake and Kinekawa, 1998), simulated gastric fluid (Herman et al. 2007), water-soluble cheese extracts (Alessandri et al., 2012), olive oil-in-water emulsion (Macierzanka et al., 2009) or water (Morisawa et al., 2009). Jakobsson et al. (1982) showed that the hydrolysis of β-LG occurred at a slower rate when it was in bovine milk or in infant formula than when it was in a purified form, althogh this effect was probably due to the buffer capacity of milk which have an impact on the enzymatic activity, alkalinizing the medium and leaving the pepsin away from its optimum pH.

Mandalari et al. (2009b) reported that the addition of PC did not affect the resistance of β-LG to gastric pepsinolysis and Macierzanka et al. (2009) studied the

combined effect of matrix and PC in digestion of β-LG showing that when the protein was in an olive oil-in-water emulsion, the presence of PC retarded hydrolysis compared with digestion without PC. It has been suggested that a lipid-induced β to α transition in the secondary structure of β-LG is accompanied by substantial disruption in tertiary structure, which is mainly driven by strong electrostatic interactions (Zhang et al., 2007). Once the tightly packed β-Lg is disrupted, hydrophobic residues could become exposed and available for insertion into a lipid bilayer, where hydrophobic interaction with the lipids may play a role in stabilizing the helical components. This could be the mechanism that provides protection against digestion in solution through the simulated duodenal digestion (Macierzanka et al., 2009).

Several studies have shown that β-LG is more sensitive to duodenal enzymes than gastric enzymes. β-LG has been directly hydrolysed with duodenal enzymes resulting in no detection of intact protein after 60 min of digestion with enzymes from human (Jakobsson et al., 1982), pig (Kitabatake and Kinekawa, 1998, Fu et al., 2002) or rat (Kitabatake and Kinekawa, 1998) origin. Other studies, although differing in the digestion model applied, used a combination of both phases (gastric followed by duodenal stage) and coincided on the fact that β-LG is degraded in the duodenal stage although they differed in the degree of hydrolysis achieved, with values ranging from complete hydrolysis (Macierzanka et al., 2009) to 3% (Bossios et al., 2011) or 72% intact protein (Dupont et al., 2010a).

It has been shown that other components present in the digestion media as bile salts may also have an influence in the results. In this sense, Gass et al. (2007) demonstrated that the addition of a bile acid mixture significantly enhance the digestion of β-LG by trypsin and chymotrypsin. In contrast, β-LG was significantly protected from

simulated duodenal digestion as a result of complex formed with the PC (Macierzanka et al., 2009). This is the reason why β-LG is most extensively degraded in infant models as compare with adult models which contains a ten-fold concentration of PC (Dupont et al., 2010a).

To date, there are only a few studies assessing the immunological potential of β-LG digestion products (Table 2). In addition, and although it can be assumed that there is a relationship between resistance to digestive enzymes and allergenicity of β-LG, in most of these studies there is a lack of correlation between digestibility and allergenicity.

There are also some *in vivo* studies about β-LG digestion showing very similar results. Scanff et al. (1990) used preruminant calves to observe the gastric digestion of raw milk, pasteurized milk and yogurt and in the three matrices studied, β-LG was resistant to proteolysis. The same results were obtained by Mahé et al. (1991) in the stomach of human adults with jejunostomy after 30 min of skimmed milk ingestion and by Kitabatake and Kinekawa (1998) using rats as a model. Bouzerzour et al. (2012) used piglet as a model of the newborn child where piglets were fed infant formula by an automatic delivery system during 28 d, and slaughtered 30, 90 and 210 min after the last meal. Contents of stomach, proximal and median jejunum and ileum were collected and characterised and the results indicated that β-LG partially resisted gastric digestion but completely disappeared in the stomach after 210 min, probably because the pH of stomach contents did not drop below 4 until 210 min after meal ingestion. Moreover, they observed that β-LG exhibited about 45% of residual IgE binding in the samples collected after 30 min in the stomach, values close to zero after 120 min and completely disappeared in the samples from jejunum and ileum.

Alessandri et al. (2012) observed no effect on the reactivity against IgE of both bovine β-LG variants, A and B, after gastric digestion of water-soluble extracts of Parmigiano-Regiano of three different maturation stages with porcine pepsin, and a little reduction of the IgE inhibition capacity after a complete simulated gastroduodenal digestion. In addition to IgE binding assays, basophil activation and *in vitro* proliferation test of sensitized PBMCs were used by Bossios et al. (2011) to quantify the residual immunoreactivity of β-LG after digestion. Their results indicated that IgE binding capacity of β-LG was not modified after gastroduodenal digestion in the presence of PC but it was reduced when digestion performed in the absence of PC, in agreement with the higher quantity of intact β-LG remaining in the digests. Overall both gastric and gastroduodenal digestion enhanced activation of sensitized basophils and proliferation of sensitized lymphocytes by β-LG.

.

Table 2. Digestibility and immunogenicity of native β-LG after an *in vitro* digestion model using porcine and bovine enzymes.

Reference	Protein	Presentation	Digestion phase										Immunologic assays	Results Immunologic assays
			Gastric						Duodenal					
			enzymes	Ratio E:S	Surfactant	pH	time (min)	Intact protein	enzymes	Ratio E:S	Bile salts	pH time (min)	Intact protein	
Alessandri et al. (2012)	β-LG A	Cheese water soluble extracts	pepsin	1:100 (v:v)	-	2.2	180	-	trypsin and chymotrypsin	1:100 (v:v)	-	7.5 240	-	IgE binding	100%
Alessandri et al. (2012)	β-LG B	Cheese water soluble extracts	pepsin	1:100 (v:v)	-	2.2	180	-	trypsin and chymotrypsin	1:100 (v:v)	-	7.5 240	-	IgE binding	100%
Morisawa et al. (2009)	β-LG (A and B)	water	pepsin	1% (v:v)	-	3	90	YES (NQ)	trypsin	1% (v:v)	-	- 60	YES (NQ)	Histamine release assay	11-85%
Bossios et al. (2011)	β-LG (A and B)	SGF	pepsin	3500 U / mg	-	2.5	60	70%	porcine pancreatic lipase	0.1% (w:v)				IgE binding	G: = / D ↓
									porcine colipase	0.055% (w:v)	7.4 mM Bile salts	6.5 60	3%	Basophil Activation	↑
									β-lg;trypsin:chymotrypsin	400:4:1 (w:w:w)				PBMC proliferation	↑
Bossios et al. (2011)	β-LG (A and B)	SGF	pepsin	3500 U / mg	PC	2.5	60	68%	porcine pancreatic lipase	0.1% (w:v)				IgE binding	G =, D =
									porcine colipase	0.055% (w:v)	7.4 mM Bile salts	6.5 60	66%	Basophil Activation	↑
									β-lg;trypsin:chymotrypsin	400:4:1 (w:w:w)				PBMC proliferation	↑

NQ: unquantified. SGF: Simulated gastric fluid. G: Gastric phase. D: Duodenal phase. PBMCs: Peripheral blood mononuclear cells.

1.3.1.3. α-lactalbumin

Regarding α-LA, the second most prevalent protein in bovine whey, Fu et al. (2002) showed it was rapidly degraded by pepsin to protein ratio of 13:1 (w:w) in SGF at pH 1.2 and no intact protein was detected after one min of digestion. Contrary to β-LG, α-LA seems to be more resistant to duodenal rather than to gastric enzymes as it was demonstrated by Fu et al. (2002). They submitted the protein to digestion with corolase PP®, a mixture of enzymes from porcine pancreas containing the pancreatic endopeptidases trypsin and chymotrypsin and various exopeptidases, and found that it was able to resist digestion for 15 min.

There are very few reports on the complete digestion of α-LA by both gastric and duodenal enzymes and there are even less studies showing the immunoreactivity and characterization of the resultant peptides. Moreno et al. (2005) carried out the digestion of α-LA using a physiologically relevant model that took into account the presence of physiological surfactants found in both milk and in the environment of the gastrointestinal tract. They showed that the interaction between PC and α-LA at gastric pH was strong enough to retard the breakdown of the α-LA during the digestion, with traces of the protein partially trimmed still evident after 1 h of digestion, while it was completely degraded in a similar model without PC. They also indicated that PC altered the fragmentation pattern of α-LA, probably because the partially unfolded state of the protein adopted at the acid pH of the stomach promotes the interaction between the hydrophobic residues of the protein and the alkyl chains of PC (Hanssens et al.,1985), hindering the access of pepsin to the cleavage sites. At the end of the duodenal stage no intact protein was detected either, with or without PC (Moreno et al., 2005). A high sensitivity to pepsinolysis has also been described by Jakobsson et al. (1982) when α-LA

was treated with human gastric fluid. In contrast, Chatterton et al. (2004) found intact protein after 1 hour of digestion of whey protein concentrate with human gastric fluid, probably due to the use of a low enzyme/substrate ratio. *In vivo* studies performed by Mahé et al. (1991) found 44% of intact α-LA in the stomach of human adults with jejunostomy 60 min after drinking skimmed milk, probably due to a higher pH in the stomach induced by the buffering capacity of milk.

In terms of immunoreactivity of the resultant products of digestion of α-LA, there are hardly any studies on the subject. Alessandri et al. (2012) observed that after gastric and duodenal simulated digestion, the IgE binding capacity of α-LA from ripened cheese water soluble extracts remained almost intact.

1.3.1.4. Minor whey proteins

Lactoferrin (LF) is a minor component of milk, and allergic reactions caused by LF are infrequent. Therefore, although it has been shown that possesses allergenic sites that may qualify it to be an allergen (Sharma et al., 2001) few studies have evaluated its allergenicity. However, and due to the high antibacterial activity of LF (López-Exposito and Recio 2006), several studies have evaluated its digestibility. Reports are contradictory with regard to the resistance of LF to hydrolysis by various commercial enzymes (Gonzalez-Chavez et al., 2009). Furlund et al. (2013) demonstrated that the *in vitro* hydrolysis of LF with pepsin, as it is the case of other milk proteins, is strongly pH dependent. They adjusted the pH of human gastric fluid either slowly or rapidly to 2.5 or 4.0 at the end of digestion and observed that a fast pH reduction to 2.5 resulted in a complete degradation in the gastric step, but a slow reduction to 2.5 or 4.0 resulted in an

incomplete degradation in the gastric step, and as a result in a delayed duodenal digestion. This pH dependence could be attributable to the different conformation of the protein at pH 4 or to the fact that pepsin is not fully active (Sreedhara et al., 2010). The results were compared with *in vivo* digestion of LF performed in 2 volunteers showing a complete hydrolysis of the protein but the authors attribute this difference to a low concentration of enzyme in the *in vitro* system used compared with that *in vivo*. In contrast, the *in vivo* gastric resistance of LF was reported previously by Troost et al. (2001). They tested the digestion of a drink containing LF in 12 healthy volunteers and the gastric survival ranged between 62% and 79%.

There are few studies about digestibility of other minor milk proteins such as BSA. Some authors demonstrated that BSA was very susceptible to pepsinolysis when porcine pepsin at pH 1.2 was used to carry the digestion (Fu et al., 2002; Herman et al. 2007). However, when human gastric fluids were used, pepsinolysis reported to be pH dependent. Chatterton et al. (2004) found intact amount of these protein when gastric digestion was performed at pH higher than 4 and complete degradation was observed only at lower pHs. IgE binding of products of digestion of BSA have been reported to decrease compare with native protein (Alessandri et al., 2012) but there are very few reports in the literature about the digestibility and allergenicity of these minor proteins making it difficult to draw any conclusions.

❖ *1.3.2 Egg proteins*

Egg white is the main source of allergens in egg. Four proteins, ovalbumin (55%), ovomucoid (11%), ovotransferrin (12%) and lysozyme (3%), named from Gal d 1 to Gal

d 4, have been identified as the major ones. Besides, other minor proteins such as ovomucin, ovoflavoprotein, avidin and ovoinhibitor have also been identified. The yolk has various proteins such as apovitelines, phosvitins and livetins which may also be allergenic, although to a lesser extent (Mine and Yang, 2008).

1.3.2.1. Ovalbumin

Several studies have evaluated OVA resistance against pepsinolysis, and even when very different enzyme to protein ratios were used, the results coincide that OVA is a protein very stable to pepsin action (Fuchs and Astwood, 1996; Dearman et al., 2002; Fu et al., 2002; Takagi et al., 2003; Thomas et al., 2004). Using a ratio that would resemble a physiological situation, 182 U/mg, the main degradation products were two hydrolysis fragments of 40.1, corresponding to Ala23-Pro385, and 4.1 kDa (López-Expósito et al., 2008; Martos et al., 2010), in agreement with digestion patterns previously described using different enzyme to protein ratios (Dearman et al., 2002; Thomas et al., 2004; Quirós et al., 2007; Takagi et al., 2003). Martos et al. (2010) went a step further and studied the effect of different gastric pHs (1.2, 2 and 3.2) on the hydrolysis of OVA with pepsin showing that the hydrolysis was much faster at the lowest pH. Moreover, they showed that the susceptibility to pepsinolysis of OVA was unmodified when PC was included in *in vitro* digestion because OVA has not enough flexibility to penetrate into PC vesicles as it has been demonstrated that OVA assumes a highly ordered molten-globule conformation at pH 2.2 which confer it a high degree of conformational stability (Tatsumi et al., 1999).

Under duodenal conditions, OVA was prone to digestion with pancreatin (Takagi et al., 2003), but in contrast, when physiological conditions were used, OVA and the previously described hydrolysis product of 40.1 kDa were very resistant to pancreatic enzymes, persisting for 120 min (Takagi et al., 2003; Martos et al., 2010). In presence of PC and bile salts, OVA and the large fragment of 40.1 kDa generated after pepsin digestion were more sensitive to proteolytic degradation, probably due to the association of the protein with the surfactants, increasing the exposure of the protein to pancreatic proteinases (Martos et al., 2010). A similar enhancement of hydrolysis in presence of bile salts was previously described by Gass et al. (2007) when β-LG digestion was performed probably through the destabilization of its tertiary structure. Bile salts could bind into the hydrophobic pockets of the protein molecule, which might destabilise the protein structure and made additional interior domains of the protein available for the protease action.

Martos et al. (2010) reported a correlation between digestibility and allergenicity of OVA, showing that the immunoreactivity against IgE was retained after *in vitro* gastric digestion, especially due to the 40.1 kDa fragment, decreasing considerably after *in vitro* duodenal digestion. Nevertheless, the residual IgE binding response after the duodenal stage suggested the presence of peptides containing intact binding sites recognizable by serum IgE.

During the storage of eggs, OVA is converted into a more heat stable form called S-OVA with a lower reactivity against IgE than native OVA (Jiménez-Saiz et al., 2012). S-OVA was more resistant to *in vitro* digestion under physiological conditions, possibly as a result of the structural changes associated to the transition of OVA into S-OVA that provide S-OVA with greater stability (Huntington and Stein, 2001). Despite the lower

IgE-binding of S-OVA, this difference was minimized after simulated gastrointestinal digestion, so that the duodenal digests of OVA and S-OVA formed *in vitro* showed a reduced but comparable reactivity towards IgE due to the similarity in their duodenal digestion patterns (Jiménez-Saiz et al., 2012).

Similarly, Maillard reaction of OVA with glucose reduced its IgE-binding but this effect was counteracted by a reduction of digestibility of the protein induced by glycation, therefore the gastroduodenal digests of glycated ovalbumin showed a similar reactivity to the digests of the native protein (Jiménez-Saiz et al., 2011a). In contrast, when OVA was mixed with pectin, gum arabic and xylan, functional biopolymers commonly used in the food industry, the reactivity towards human IgE was considerably increased as well as the resistance to digestion (Jiménez-Saiz et al., 2013). Polovic et al. (2009) reported the existence of a protective matrix effect on the digestion as polysaccharides. Gels can provide a physical obstacle to the mobility of enzymes and substrates. Similarly, OVA attached to palmitoyl residues has also been reported to remain longer in the stomach when compared with native OVA (Oliveira et al., 2007). These results underline the importance of the food matrix in both the digestibility and the potential ability to trigger an immune response of OVA. In this regard, Martos et al. (2013) investigated the existence of matrix effects on the proteolytic stability and resultant IgE-binding of egg allergenic proteins. The presence of egg yolk slightly increased the susceptibility to digestion of OVA, but the gastric and duodenal digests had comparable IgE-binding capacities. On the other hand, Jiménez-Saiz et al. (2012) showed that the presence of OVA as part of an emulsion, increased its digestibility resulting in a slightly lower IgE-binding capacity of the *in vitro* gastric and duodenal digests compared to those obtained from the protein in solution.

1.3.2.2. Ovomucoid

Ovomucoid (OM) is a protein highly sensitive to proteolytic degradation. Kovacs-Nolan et al. (2000) reported a rapid degradation of OM during *in vitro* gastric digestion with the formation of large fragments exhibiting reduced IgE-binding activity as compared with the native protein. Little changes in the digestion pattern were observed during the duodenal phase, probably because the peptides released by pepsin action retain trypsin inhibitory activity that helps to maintain OM peptide fragment integrity. A later study demonstrated that after *in vitro* gastric and duodenal digestions, the immunoreactivity of OM towards IgE from egg-allergic patients was reduced to a great extent (Jiménez-Saiz et al., 2011a) although it still maintained residual IgE binding in accordance with previous findings (Matsuda et al., 1985; Takagi et al., 2005). Moreover it has been showed that *in vitro* digested OM has a diminished basophil activation capacity when compared with the intact protein (Martos et al., 2011).

Chemical modifications could affect the structure of OM modifying its susceptibility to gastrointestinal digestion. Kovacs-Nolan et al. (2000) showed that reducing the disulphide bonds in OM enhanced its digestibility and decreased its allergenicity *in vitro* (Kovacs-Nolan et al., 2000). On the contrary, it has been shown that Maillard reaction with glucose did not affect the digestibility of OM, although it increased the immunoreactivity with respect to the intact protein (Jiménez-Saiz et al., 2011a).

1.3.2.3. Lysozyme

The information available related to the behaviour of lysozyme (LYS) towards gastrointestinal digestion is limited and sometimes contradictory due to the employment

of different digestion models. Mine et al. (2004) reported the complete hydrolysis of LYS after 60 min of treatment with pepsin at pH 1.0, while other authors reported that LYS is resistant to pepsin action (Fu et al., 2002; Polverino de Laureto et al., 2002). Ibrahim et al., (2005) found up to 60% of intact protein after 120 min of digestion with pepsin at pH 4. Later experiments carried out by Jiménez-Saiz et al. (2011b) using physiological conditions showed that *in vitro* gastric digestion of LYS was extremely pH dependent and at values equal to or higher than 3.2, there was no hydrolysis of the protein, even at pH 2 intact protein was still found at the end of the gastric phase. LYS was only completely degraded at pH 1.2. The presence of 4 disulphide bonds in the structure of LYS provided a great stability to the protein and the presence of PC, slightly increased LYS resistance to digestion (Jiménez-Saiz et al., 2011b), likely due to the association between LYS and PC films (Mudgil et al., 2006).

LYS may also be resistant to trypsin and chymotrypsin hydrolysis or just slightly hydrolysed, as it has been reported by During et al., (1999) and Mine et al., (2004) respectively. This resistance could be enhance due to the precipitation of LYS in the presence of bile salts impairing its hydrolysis by duodenal enzymes (Jiménez-Saiz et al., 2011b). Probably, precipitation of LYS is caused due to an electrostatic interaction with negatively charged bile salts at pH 6.5 (Jiménez-Saiz et al., 2011b). Therefore, in a duodenal environment, intact LYS could be absorbed, as it has been demonstrated by Takano et al. (2004), with the possibility of eliciting and allergic reaction. On the other hand, the presence of PC partially avoided LYS precipitation (Jiménez-Saiz et al., 2011b), suggesting a positive effect on solubilisation of the mixed bile salt-PC micelles present in the duodenal medium (Mandalari et al., 2009).

A matrix effect has also observed by Jiménez-Saiz et al. (2012), who described a high resistance of LYS to hydrolysis by pepsin when it was included in egg solution rather than in egg emulsion, probably because of a higher exposure of the protein to the enzyme.

Regarding to immunoreactivity of the products generated during digestion, Jiménez-Saiz et al. (2011b) reported that gastric digests of LYS showed high IgE-binding capacity using sera from egg-allergic patients but there is no more available information in the literature related to this issue.

1.4. FUTURE PROSPECTS

Significant progress has been made in the study of digestibility and allergenicity of food proteins. A multitude of methods of different complexity, biological significance and requirements now exist to increase knowledge in this topic. However, some questions such as if *in vitro* digestibility is a good predictive tool for the evaluation of protein allergenicity, or which digestion model should be used, remain unresolved. Any factor that potentially affects protein digestibility could modify the proteolysis pattern of a protein during the digestion process and therefore change its capacity to induce or trigger an allergic reaction. This fact highlights the need to establish standardized assay conditions, so that digestibility results can be compared between different studies. Also, criteria need to be established to relate *in vitro* digestibility to allergenic potential. Ideally, the combination of immunological assays, such as IgE binding capacity, with *in vitro* gastrointestinal digestion models simulating physiological conditions must be required to study the potential allergenicity of food proteins. More research is needed to understand

the underlying mechanism of food allergy and to understand the relationship between protein digestibility and allergenicity.

2. OBJECTIVES

Cow's milk allergy is defined as an immunologically mediated adverse reaction to cow's milk protein and it is usually the first food allergy identified in childhood. With an incidence that ranges from 1.2 to 17% in population-based studies in different countries, allergy to cow's milk often precedes the development of other IgE-mediated allergies. Although most children outgrow cow's milk allergy by the age of 4 years, a significant proportion of the population retains the allergy throughout life. Similarly, egg allergy is one of the most frequent food allergies in children below the age of three, with common symptoms frequently involving the skin as well as the gut and, in more severe cases, anaphylaxis. A meta-analysis of double-blind placebo-controlled food challenges confirmed a prevalence between 0.2 and 7%, with most children not outgrowing their egg allergy by school age but, instead, showing a long progression to egg allergy resolution.

One of the main aspects to consider when evaluating the allergenic potential of food proteins is the effect of gastrointestinal digestion. It is known that allergens are usually able to survive the harsh acidic environment of the stomach, tolerate the presence of surfactants and resist digestion by gastrointestinal enzymes, in order to reach the gut associated lymphoid tissue. They might also be digested into high molecular weight peptide fragments, which retain the same, or sometimes increased, IgE binding and T-cell stimulating capacities. So far, the studies investigating the gastrointestinal stability of the main food allergens have been performed *in vitro* by the use of enzymes of bovine or porcine origin as part of different digestion models, ranging from simple one-step hydrolyses, to advanced physiologically relevant systems where subsequent gastric and duodenal digestions are conducted under conditions that mimic the *in vivo* processes in infants and adults. These latter studies have highlighted the effect that the enzyme to substrate ratio, pH and concentration of physiological surfactants, such as

phospatidylcholine and bile salts, exert on the gastrointestinal stability of OVA and their influence in the resulting immunoreactivity of the digests. However, these relevant parameters regarding digestion, such as enzyme activity, volume of digestive juices secreted, pH or surfactants level, vary widely among individuals and also with the type and amount of food ingested and the time of the day, making *in vivo* conditions very difficult to simulate. In addition, human gastrointestinal juices contain complex mixtures of enzymes which could present either broader or different specificities or vary in their functional parameters with respect to purified commercial enzymes from animal sources.

Given the importance of the resistance of food proteins to gastrointestinal digestion in their capacity to modulate the immune response, the aim of this work was to compare the digestibility of **β-CN**, **β-LG** and **OVA**, considered relevant cow's milk and egg allergens, in two different *in vitro* systems using simulated and human fluids, and to assess the immunochemical properties of the resulting digests. To this aim, the products of protein digestion in both systems were identified by RP-HPLC-MS/MS and the IgE binding epitopes were characterized. In addition, the influence of gastrointestinal digestion on the immunological properties was further assessed in two major allergens of egg white: **OM** and **LYS**. In the first case, particular attention was paid to elucidate the role of the carbohydrate moieties of OM in its allergenic properties. In the second case, and since no relevant LYS IgE-binding epitopes had been previously reported, it was attempted to identify the main LYS IgE-binding areas following proteolysis, either mimicking gastrointestinal digestion or with papain, which is used together with LYS in LYS-containing drugs.

3. MATERIALS AND METHODS

3.1. MATERIALS

❖ *3.1.1. Proteins*

β-CN (EC 232-555-1) and β-LG (EC 232.928.9) from bovine milk and OVA (EC 232-692-7), OM (EC 232-906-9) and LYS chloride (Grade VI, ~60000 units/mg protein, EC 3.2.1.17) from chicken egg white were purchased from Sigma-Aldrich (St. Louise, MO, USA).

❖ *3.1.2. Human digestive fluids*

Saliva was collected from 3 healthy donors after 2 h fasting. Human gastric and duodenal fluids were collected by gastroscopy from 4 different patients without any gastrointestinal pathology at the Hospital Universitario La Paz (Madrid, Spain) after 8 hours of fasting. Aspirates were collected on ice and gastric and duodenal fluids were separately pooled and frozen to stop any enzymatic reaction. Pepsin activity was assayed spectrophotometrically as recommended by Sigma with haemoglobin as a substrate (Anson, 1938). The activities of trypsin and chymotrypsin were also measured spectrophotometrically following a continuous rate determination recommended by Sigma, using N-benzoyl-L-arginine-ethylester (BAEE) and N-benzoyl-L-tyrosine-ethylester (BTEE) as substrates respectively (Bergmeyer et al., 1974; Wirnt, 1974). The determinations showed a pepsin activity of 728.9 U/mL in the pooled gastric fluid (pH 2.13) and trypsin and chymotrypsin activities of 167.2 and 2.8 U/mL, respectively, in the duodenal fluid (pH 7.8). The bile salt concentration in the duodenal fluid was 0.7 mM, as estimated by a commercial bile acids determination kit from Trinity Biotech (Bray, Wicklow, Ireland).

❖ *3.1.3. Human sera*

Individual serum samples from children with proven allergy to bovine milk and egg proteins were collected from the Hospital Infanta Sofia (San Sebastián de los Reyes, Madrid, Spain), the Hospital Infantil Universitario Niño Jesús (Madrid, Madrid, Spain) and the Mount Sinai Medical Center (New York, NY, USA). The diagnosis of IgE-mediated milk and egg allergy was made by an allergist on the basis of objective symptoms of an acute reaction after milk or egg ingestion, together with evidence of specific IgE antibodies against milk or egg. The specific IgE levels of the sera used are shown in Annexes 1-5. Serum samples from non-allergic donors were also used.

Ethics statement: All human samples were obtained with written consent of the patients. All experiments were approved by the Bioethics Committee from the Consejo Superior de Investigaciones Científicas (CSIC), Spain.

3.2. METHODS

❖ *3.2.1. Sample preparation*

3.2.1.1. Ovomucoid enzymatic deglycosylation

OM was dissolved in 5 mM potassium phosphate, 4 mM $CaCl_2$, 0.04 % NaCl, pH 7.5, at a concentration of 20.7 mg/mL and deglycosylated with PNGase F (500 U/mL, Sigma-Aldrich, 1 U/0.8 mg of OM) at 37 °C, with constant stirring for 24h. PNGase F was added again to the solution at the same ratio and incubated for a further 4 days at 37 °C. A control without PNGase F was also included. Free sugars were removed from deglycosylated OM (dOM) by centrifugation at 4000g for 5 min at 4 °C in ultrafiltration

devices of 10.000 Da cut off from Millipore (Billerica, MA, USA), until no sugars were detected in the permeates according to the phenol-sulfuric acid method (Masuko et al., 2005). Briefly, 50 µL of permeate were added to a 96-well microplate and 150 µL of concentrated sulphuric acid was added rapidly to cause maximum mixing, followed by 30 µL of 5% phenol (Sigma-Aldrich) in water. After incubating for 5 min at 90ºC in a static water bath, the plate was cooled to room temperature and the absorbance at 490 nm recorded with a Multiskan FC microplate reader from Thermo Scientific (Waltham, MA, USA). The concentration of deglycosylated ovomucoid was measured spectrophotometrically (490 nm) by the Pierce® BCA Protein Assay Kit from Pierce Scientific (Rockford, IL, USA) following the manufacturer´s instructions.

3.2.1.2. Reduction and alkylation of lysozyme

LYS was reduced and alkylated as previously described (Mine and Zhang, 2002a). A solution of 1 mg/mL of LYS in denaturation buffer (6 M guanidinium hydrochloride in 0.6 M Tris-HCl, pH 8.6) was prepared. Then, an equal volume of 4 mM 1,4-dithiothreitol (Sigma-Aldrich) was added to give a final concentration of 2 mM and 400 µL of iodoacetic acid (Sigma-Aldrich) were added dropwise with stirring in the dark. The sample was incubated in the dark for 1 h at 37 °C followed by dialysis against 50 mM ammonium bicarbonate, pH 7.8, for 24 h. The protein content was determined with the BCA Protein Assay Kit from Pierce Scientific.

3.2.1.3. Hydrolysis of lysozyme with papain

LYS was hydrolysed with papain (76218, Papain from Carica papaya, 12 U/mg, EC 232.627.2, Sigma-Aldrich) for 3 h at 54ºC at an enzyme to substrate ratio of 2:5

(w:w), similar to that found in Lizipaina®, a drug employed for the symptomatic relief of mild conditions of the throat and mouth. The reaction was stopped by heating at 100ºC for 10 min.

❖ *3.2.2. Digestion models*

3.2.2.1. In vitro digestion with simulated fluids

Preparation of phospholipid vesicles

Phospholipid vesicles were prepared by dissolving egg L-alpha-phosphatidylcholine from Larodan (Limhamn, Malmo, Sweden) in simulated gastric fluid (35 mM NaCl pH 2.0) at a concentration of 9.58 mg/mL. Then, the solution was sonicated in ice (raising the power from 10% to 50% in 5 min, and keeping it 5 min at 60% power), not exceeding a sample temperature of 40ºC, following Martos et al. (2010). Phospholipid vesicles were filtered through Filtropur 0.45 µm polyethersulfone from Sarstedt (Nümbrecht, North Rhine-Westphalia, Germany) to remove any possible titanium particles.

Oral digestion

Proteins were dissolved in simulated saliva fluid (potassium phosphate 0.005 M, $CaCl_2$ 0.004 M, NaCl 0.04 %, pH 6.5) at a concentration of 46 mg/mL. After incubation at 37 °C for 15 min, a solution of α-amylase (EC 3.2.1.1, 210 U/mg solid, Sigma-Aldrich) was added at the physiological ratio of 150 U/mL (Bernard, 1953) of simulated saliva fluid. *In vitro* oral digestions were performed at 37 °C in an incubator with moderate shaking and aliquots were taken after 2 min. The digestions were stopped by decreasing the pH to 3.5 with 1N HCl.

Gastric digestion

In vitro gastric digestions were performed either using the 2 min-oral digests as the starting material, previously adjusted to pH 2.0 with 1N HCl, or using the proteins dissolved in simulated gastric fluid. Samples were mixed with the phosphatidylcholine vesicle solution (1:1.2, v:v), and after incubation at 37 °C for 15 min, pepsin (EC 3.4.23.1, 3640 U/mg protein, Sigma-Aldrich) dissolved in simulated gastric fluid pH 2.0 was added at a physiological enzyme to substrate ratio (1:20, w:w) (182 U/mg of protein) (Moreno et al., 2005). The gastric digestions were performed at 37 °C in an incubator with moderate shaking and aliquots were taken at different time-points from 0 to 60 min. The digestions were stopped by increasing the pH to 7.5 with 1M $NaHCO_3$ to irreversibly inactivate pepsin, giving a final concentration of 5.3 mg/mL of protein and 4.8 mg/mL of phosphatidylcholine.

Duodenal digestion

Duodenal digestions were performed on the 60 min gastric digests, re-adjusted to pH 6.5, with the addition of 0.25 M Bis-tris, pH 6.5 (Sigma-Aldrich), 1 M $CaCl_2$ (7.6 mM final concentration) and a 0.25 M bile salt mixture containing equimolar quantities of sodium glycodeoxycholate and sodium taurocholate (Sigma-Aldrich) (7.4 mM final concentration of bile salt). After preheating at 37 °C for 15 min, pancreatic porcine lipase (EC 232-619-9; type VI-S, 47900 U/mg protein, Sigma- Aldrich), pancreatic porcine colipase (EC 259-490-1; Sigma-Aldrich), pancreatic bovine trypsin (EC 232-650-8, type I 10100 BAEE U/mg protein, Sigma-Aldrich), and pancreatic bovine a-chymotrypsin (EC 232-671-2; type I-S; 55 U/mg protein, Sigma-Aldrich), were added to the duodenal mix at enzyme to substrate ratios of 24.7 U/mg protein, 1:895 (w:w), 34.5 U/mg protein and 0.44 U/mg protein respectively (Moreno et al. 2005). The reactions were carried out at 37 °C

for different periods from 0 to 60 min and stopped by adding a solution of Bowman–Birk trypsin-chymotrypsin inhibitor from soybean (T9777; Sigma-Aldrich), at a concentration calculated to inhibit twice the amount of trypsin and chymotrypsin present in the digestion mix. The final composition of the mixture was 3.3 mg/mL phosphatidylcholine, 3.9 mg/mL protein, 7.4 mM bile salt mixture, 7.6 mM $CaCl_2$ and 20.3 mM Bis-tris.

3.2.2.2. In vitro digestions with human fluids

In vitro protein digestion with human fluids was performed in 3 steps, simulating the oral, gastric and duodenal phases. First, protein was dissolved in saliva (20 mg/mL, pH 6.5) and incubated at 37°C for 2 min, followed by a pH decrease to 3.5 with 1N HCl. For gastric digestions, 112 μL of the protein solution (20 mg/mL) were mixed with 560 μL of the pool of human gastric fluids at pH 2.1, so that the pepsin to protein ratio was the same as that employed in the digestion with commercial pepsin (182 U/mg). After 1 hour of gently shaking at 37°C, the pH was adjusted to pH 6.5 with 1 M $NaHCO_3$ and 300 μL of the gastric digest were mixed with 198 μL of the pool of human duodenal fluids at pH 6.7, so that the enzyme to substrate ratios were 34.5 U/mg of trypsin and 0.49 U/mg of chymotrypsin. Aliquots were taken at different time-points during the incubation. The reaction was stopped with a solution of Bowman–Birk trypsin-chymotrypsin inhibitor from soybean as described above.

❖ *3.2.3. Sample characterization*

3.2.3.1. Circular Dichroism

Circular dichroism spectra were obtained in a Jasco J-810 spectropolarimeter from Jasco Corporation (Tokyo, Japan). Far (195-260 nm) and near (250-350 nm) ultraviolet circular dichroism spectra of samples, in phosphate buffer 50 mM pH 7.0, were recorded at 20 °C using cells with respective path lengths of 0.1 and 0.2 cm. Spectra represent the average of three accumulations collected at 20 nm/min, with a 2 s time constant, a 0.2 nm resolution, and a sensitivity of 100 mdeg. The samples were dissolved at 0.2 mg/mL for the analysis in the far ultraviolet region and at 0.5 mg/mL for the near ultraviolet region. The buffer blanks were subtracted from each circular dichroism spectrum. Empirical determinations of protein secondary structure were obtained employing the CDNN secondary structure analysis software from Applied Photophysics Ltd (Leatherhead, Surrey, UK).

3.2.3.2. Electrophoresis SDS–PAGE

SDS–PAGE analyses were performed using Bis-Tris or Tris-Tricine acrylamide gels from Bio-Rad Laboratories (Richmond, CA, USA). Samples were diluted 1:4 (v:v) in 0.5 M Tris-HCl buffer, pH 6.8, containing 1.6% SDS, 8% glycerol, 4% 2-β-mercaptoethanol and 0.002 % bromophenol blue or in Tricine sample buffer (Bio-Rad), respectively, and heated at 95 °C for 4 min. Electrophoresis were carried out at 150V in XT running buffer MES, when Bis-Tris gels were used, and at 100V in Tris-Tricine running buffer, when Tris-Tricine gels were used. A molecular mass marker ranging in molecular mass from 10 to 250 kDa was used, when the electrophoresis was carried out

using the Bis-Tris gels (Precision Plus Protein standards, Bio-Rad), and ranging from 1.42 to 26.62 kDa, when the Tris-Tricine gels were employed (Precision Plus Protein Kaleidoscope standards, Bio-Rad). Gels were stained with Coomassie Blue G-250 (Bio-Rad) or Periodic Acid Schiff. For the **Coomassie blue** staining, proteins were fixed with a solution of 40% methanol (v/v) and 10% acetic acid (v/v) for 30 min. Afterwards, the gels were incubated with Bio-SafeTM Coomasie G-250 (Bio-Rad) overnight and rinsed with water until the background was clear. **Periodic Acid Schiff** staining was performed by fixing the protein bands with 12.5% trichloroacetic acid (w/v) for 30 min. After removing the excess of acid by washing with water, a solution of 1% (w/v) periodic acid in 3% (v/v) acetic acid was added. Excess periodate ions were removed by washing with water and the color was developed by dipping the gel in Schiff's reagent for 1 h in the dark. Finally, the gel was washed with a solution of 0.5% (w/v) $Na_2S_2O_5$ and rinsed with water.

3.2.3.3. Reversed Phase-High Performance Liquid Chromatography (RP-HPLC)

Native proteins and their hydrolysates, at a concentration of 3.9 mg/mL, were analysed in a Waters 600 HPLC system using a RP318 C18 column (250x4.6 mm, 5 µm of particle size, 300 A pore size, Bio-Rad). The injection volume was 50 µL and the digests were eluted by using 0.37% (v/v) trifluoroacetic acid from Scharlau Chemie (Barcelona, Spain) in double-distilled water as solvent A, and 0.27% (v/v) trifluoroacetic acid in HPLC-grade acetonitrile from Lab-Scan (Gliwice, Poland) as solvent B. A linear gradient of solvent B in A ranging from 0% to 60% in 60 min was used to fractionate the peptides contained in the samples at 1 mL/min followed by washing 35 min with 60% of solvent B and 10 min with 100% of solvent B. Detection was carried out at 220 nm and

data were processed by using Empower 2 Software from Waters Corporation (Milford, Massachusetts, USA).

3.2.3.4. Peptide sequencing by RP-HPLC-MS/MS

RP-HPLC-MS/MS analyses of the digested samples were performed on an Agilent 1100 HPLC System (Agilent Technologies, Waldbronn, Baden-Württemberg, Germany) with a HiPore column from Bio-Rad (RP318 C18 column 250x4.6 mm, 5 mm of particle size). The HPLC system was connected on-line to an Esquire 3000 quadrupole ion trap equipped with an electrospray ionisation source from Bruker Daltonik (Bremen, Bremen, Germany). The mobile phases used were 0.37% (v/v) trifluoroacetic acid in double-distilled water as solvent A, and 0.27% (v/v) trifluoroacetic acid in HPLC-grade acetonitrile as solvent B. A linear gradient of solvent B in A ranging from 0% to 60% in 60 min was used to fractionate the peptides contained in samples. The injection volume was 50 µL and the flow rate 0.8 mL/min. Mass spectra were acquired over the range 100-3000 m/z. Using Data Analysis™ version 4.0 from Bruker Daltonik, the m/z spectral data were processed and transformed to spectra representing mass values. BioTools version 3.1 (Bruker Daltonik) was used to process the MS(n) spectra and to perform peptide sequencing. To aid the identification of disulphide linked fragments, the samples were also analysed after a reducing step using 70 mM dithiothreitol (DTT), at pH 7.0, for 1 h at 37ºC.

3.2.3.5. MALDI-TOF/TOF MS

Size distribution

The range of peptide masses in the protein digests was analysed by MALDI-TOF/TOF MS using a Bruker Autoflex Speed spectrometer (Bruker Daltonik). The sample (0.5 μL) was loaded on a dry DHB matrix spot (0.5 μL of 20 mg/mL 2,5-dihydroxybenzoic acid in acetonitrile/H_2O/ trifluoroacetic acid (66/33/0.1) (v/v/v) onto a Bruker Anchorchip target and allowed to dry. All mass spectra were initially calibrated with Peptide Calibration Standard and Protein Calibration Standard I (Bruker Daltonik).

Analyses of the peptide electrophoretic bands

The identification of the exact sequence of the IgE reactive peptides detected by Western blotting was done by mass fingerprinting and MALDI TOF/TOF analysis of tryptic digests, in combination with intact molecular weight determination. Analyses were performed on an Autoflex SpeedTM (Bruker Daltonik). Bands were manually excised from gels and in-gel digested with trypsin from Promega corporation (Fitchburg, MA, USA), at 20 ng/mL in NH_4HCO_3 25 mM, with or without previous reducing and alkylation steps using dithiothreitol and iodoacetamide (Shevchenko et al., 2006). After digestion, the supernatants were collected, cleaned with a C18 Zip Tip pipette tips (Millipore, Billerica, MA, USA) and spotted onto a MALDI target plate with a DHB matrix. For peptide identification, Biotools version 2.1 (Bruker Daltonik) was used. Search parameters were carbamidomethyl cystein and oxidized methionine as variable modifications, peptide mass tolerance 500 ppm, and MS/MS fragments tolerance 0.5 Da.

For intact molecular weight determination, proteins were electroblotted and directly analysed after dissolving the nitrocellulose membrane in MALDI matrix solution, prepared as a saturated solution of α-CHCA (alpha-cyano-4-hydroxy-cinnamic acid) in

acetonitrile/methanol 70/30%, containing 1% trifluoroacetic acid (Martos et al., 2012). Samples were spotted on a Bruker Anchorchip target and spectra were acquired on linear mode.

❖ *3.2.4 Sample immunoreactivity*

3.2.4.1. Western Blotting

After SDS-PAGE separations on Precast Criterion gels, the gels were soaked in transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, pH 9.2) for 20 min and subjected to semidry transfer in a Trans-Blot SD (Bio-Rad) for 30 min at 18 V. Transfer effectiveness was assessed by staining the membrane with Ponceau reagent. Then, the nitrocellulose membranes were blocked with Tris Buffer Saline (50 mM Tris, 150 mM NaCl, pH 7.6) with Tween 20 (0.05% v/v) (TBST), containing 1% (w/v) bovine serum albumin (BSA) for 60 min. The membranes were washed with TBST and incubated overnight at 4ºC with human sera diluted in TBST containing BSA 0.1% (w/v). After a new washing-step, the membranes were incubated overnight at room temperature with biotin conjugated antihuman IgE antibody clone HP6029, from Southern Biotech (Birmingham, AL, USA) diluted 1:500 in TBST containing 0.1% (w/v) BSA. After a new washing-step, they were incubated with HRP conjugated streptavidin for 1 h at room temperature. Finally, the membranes were rinsed and detection with chemiluminescence was developed with Amersham TM ECL TM Prime from GE Healthcare (Pittsburgh, PA, USA). Image acquisition was performed using the VersaDoc Imaging System (Bio-Rad).

3.2.4.2. Human IgE Binding by Inhibition ELISA

Human IgE binding to LYS and its digests was assessed by inhibition ELISA as previously reported Jiménez-Saiz et al. (2013). Single wells of polystyrene Corning Costar microtiter plates (Lowell, MA, USA) were coated with 10 µg/mL of protein solution in 0.01M phosphate-buffered saline (PBS), pH 7.4, and incubated overnight at 4ºC. The plates were washed three times with PBS containing 0.05% Tween 20 (PBST). Residual free binding sites were blocked by incubation with PBS containing 2.5% Tween 20 for 4 h at room temperature. Then, serial dilutions of each sample (not less than 10) were incubated during 120 min with sera previously diluted in PBST (1:1 v/v) and, after washing the plate, 50 µL was added to each well. Each sample was added in duplicate and a negative control without serum (native protein in PBST) and positive controls (sera diluted in PBS) were included in each plate. After 120 min of incubation, 50 µL of polyclonal rabbit anti-human IgE from Dako (Glostrup, Hovedstaden, Denmark) diluted 1:1,000 in PBST, were added per well and incubated for 60 min. Then, 50 µL of polyclonal swine anti-rabbit immunoglobulin conjugated with horseradish peroxidase (HRP) (Dako), diluted 1:2,000 in PBST, were added per well and incubated for 60 min. Before the addition of the enzyme substrate, a signal amplification system based on the subsequent addition of tyramide-biotin and streptavidin-HRP, was used following the instructions of the manufacturer (PerkinElmer, Walthman, MA, USA). Finally, 50 µL of 3,3'-5,5'-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich) were added to each well and after 30 min of incubation, the reaction was stopped with 0.5 M sulphuric acid. Absorbance was measured at 450 nm on an automated Multiskan FC microplate reader (Thermo Scientific). A nonlinear adjustment of the data obtained for each dilution was applied for each serum and sample. The adjustment model was a sigmoidal dose-response

curve with variable slope, from which the EC50 (the concentration that binds 50% of seric IgE) was obtained with the program GraphPad Prism version 5 (La Jolla, CA, USA). The IgE binding capacity was expressed as the percentage of the EC50 of the intact protein.

3.2.4.3. Microarray

Peptides, slides, printing

The peptide microarray immunoassay was carried out at the Mount Sinai School of Medicine in New York as previously described Shreffler et al. (2005) and Lin et al. (2009) with minor modifications. Among the peptides identified from the gastric and gastroduodenal phases of digestion, the peptides shorter than 8 amino acids, that which were part of longer sequences and the peptides that overlapped with well-established epitopes for β-CN and β-LG by microarray and SPOT membrane in previous studies were discarded. Peptides with 8 and 9 amino acids were extended to 10 amino acids with the preceding or following amino acids of the protein chain and the peptides over 20 amino acids were replaced by 2 shorter overlapping peptides. Peptides selected from *in vitro* digestions, together with a library of peptides consisting of 20 amino acids overlapping by 17 (3-offset) corresponding to the primary sequence of β-CN and β-LG, were commercially synthesized by JPT peptide Technologies (Berlin, German). Peptides were diluted 1:2 in protein printing buffer from Arrayit (Sunnyvale, CA, USA) and printed in 2 sets of triplicates on epoxy-derivatized glass slides (Arrayit) by using the NanoPrint Microarrayer 60 (Arrayit). Protein printing buffer alone, used as a negative control and for background normalization, and fluorochrome-labeled BSA, used for the purpose of grid alignment during analysis, were included on each slide.

Immunoassay

The printed slides were blocked with 400 mL of 1% human serum albumin (HAS) in PBST for 60 min at 31°C, followed by incubation with 250 µL of each patient's serum diluted 1:5 in PBST-HSA overnight at 4°C. For IgE detection the slides were incubated overnight at 4°C with 250 µL of a cocktail of 3 biotinylated monoclonal anti-human IgE antibodies, one from Invitrogen (Carlsbad, California, USA) diluted 1:250, one from BD Biosciences (San Jose, California, USA) diluted 1:250 and one from Phadia (Upsala, Upsala, Sweden) diluted 1:1000. Slides were then incubated for 3 hours at 31°C with 200 µL of a cocktail of anti-biotin dendrimer Oyster 550 (Genisphere, Philadelphia, PA, USA) in dendrimer buffer (Genisphere) at 0.6 mg/mL with the addition of 0.02 mg/mL salmon sperm DNA (Invitrogen). All incubations were performed in the dark in a humidity chamber, followed by washing with PBS, PBST and 15mM Tris buffer pH 8.0 for different times. Following the last washing, the slides were centrifuge dried and scanned with a ScanArrayGx (PerkinElmer).

Data analysis

The fluorescence signal of each spot was digitalized with the program ScanArray Express (PerkinElmer) and transformed to a z score, as previously described (Lin et al. 2009). An index z value of each peptide element was generated from the median of z scores of the 6 replicate spots. An individual peptide sample was considered positive if its z score exceeded 3, meaning that the signal was significantly above the background ($P < 0.003$).

3.2.4.4. Dot-Blot

Peptides

Peptides selected from *in vitro* digestions, following the same criteria as described above, were commercially synthesized (JPT peptide Technologies).

Immunoassay

The nitrocellulose membranes were conditioned in Tris buffer (48 mM Tris, 39 mM glycine, 20% methanol, pH 9.2) for 20 min, and 1µl of each peptide was spotted onto the membrane and allowed to dry. Then, the nitrocellulose membranes were blocked with TBST with 1% w/v BSA, for 60 min and washed with TBST. Immunolabelling with ten individual serum samples was conducted as described for the Western Blotting experiments.

3.2.4.5. Basophil activation test

Basophil activation assays were performed as described by Martos et al. (2011), with some modifications. Peripheral blood mononuclear cells (PBMCs) from egg-tolerant adult donors were isolated according to standard procedures (Movérare et al., 2000). Briefly, blood was diluted 1:1 in PBS and gently layered onto a conical tube with Ficoll. After centrifugation at 300g for 30 min using slow acceleration and without deceleration to prevent mixing of the liquids, the buffy coat, containing the PBMCs, was aspirated and washed with PBS. PBMCs were stripped from bound IgE by treatment with lactic acid (13.4 mmol/L lactate, 140 mmol/L NaCl, 5 mmol/L KCl, pH 3.9) for 5 min on ice and re-sensitized with a 1:1.5 dilution of a pool of sera from children with egg allergy. Subsequently, cells were incubated with the samples at a range of concentrations from 0.01 to 10 µg/mL. Cells were stained for CD63, CD123 (BD Biosciences), HLA-DR, and

CD203c from Beckman Coulter (Indianapolis, IN, USA), fixed with BD FACS™ Lysing Solution (BD Biosciences), and acquired in a Gallios Flow Cytometer (Beckman Coulter). Dissociation of IgE with lactic acid and successful re-sensitization, as assessed by detection of cell surface IgE using monoclonal anti-human IgE, showed an almost complete loss and recovery of bound IgE (94.3, 0.72, and 95.8% of IgE-coated basophils in nonstripped, stripped, and re-sensitized cells, respectively). Anti IgE antibody (Dako) and fMLP (Sigma-Aldrich) were used as positive controls. Percentage of activation upon stimulation with the samples was calculated and normalized according to the value obtained for RPMI-IL3 (negative control).

3.2.4.6. Proliferation of PBMCs

PBMCs from egg-allergic patients were cultured at a concentration of $2x10^6$ viable cells/mL in a 96-well microplate and stimulated with the samples at 20 and 5 μg/mL for 5 days at 37ºC. PBMCs proliferation was determined using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay following Keller et al. (2006). Briefly, 100 μL of MTT (0.5 mg/mL) in PBS was added to the microplate and incubated 3 hours at 37ºC. Afterwards, the plate was centrifuged at 600g for 10 min and the pellet was dissolved in 1:1 of dimethylsulfoxide:methanol (v:v). Absorbance was measured at 570 and 650 nm on an automated Multiskan FC microplate reader (Thermo Scientific). PBMCs from egg-tolerant donors were also employed. The results were considered positive when the stimulation index (SI) was higher than 1.5. SI on MTT assay was calculated according to the formula:

$$SI = \frac{\text{OD (570-650 nm) of stimulated cells}}{\text{OD (570-650 nm) of non-stimulated cells}}$$

3.2.4.7. Skin tests and measurement of IgE and IgG4

The skin prick test, serum total and specific IgE, and serum specific IgG4 to different egg protein fractions, shown in section 4.5.2., were assessed before and after 15, 30 and 90 days of oral immunotherapy of a 15-year-old boy at the Hospital Infantil Universitario Niño Jesús (Madrid).

❖ *3.2.5. Statistical analysis*

All data was analyzed with GraphPad Prism version 5. Student t-test was used for comparison between groups. Data were considered statistically significant if $P<0.05$.

4. RESULTS AND DISCUSSION

β-CASEIN

4.1. *In vitro* digestibility of bovine β-Casein with simulated and human oral and gastrointestinal fluids. Identification and IgE-reactivity of the resultant peptides

Caseins (up to 80% of the total milk protein content) are one of the most critical milk allergens accounting for the highest circulating IgE levels (Restani et al., 2009). Among them, β-casein (β-CN, Bos d 8) (approximately 27% of the total milk proteins) represents a serious health risk to patients with cow's milk allergy, since 75% of the sera from patients with IgE-mediated allergy to cow´s milk have IgE directed against β-CN (Shek et al., 2005).

β-CN is a protein with a molecular weight of 24,000 Daltons and 209 amino acids, including 35 proline residues uniformly distributed in the molecule. This large amount of proline prevents the formation of α-helix, β-sheet and β-turns and gives it a very flexible three-dimensional structure with large disordered areas (Dunker et al., 2001). It also contains 5 phosphoserine residues in the N-terminal end (being phosphorylation highly variable depending on the variant gene) and no cysteine residues. These structural characteristics prevent β-CN from undergoing an abrupt transition from one type of conformational state to another, and its polypeptide chain adopts a set of secondary structures which are in equilibrium with each other. Therefore β-CN has a very stable structure which could protect many potential epitopes when it is subjected to heating (Holt and Sawyer, 1993) or even to hydrolysis with proteases during the digestion process.

There are few published studies describing β-CN digestion with physiologically relevant models using commercial enzymes (Dupont et al., 2010a, 2010b). Alternatively, other authors have used human gastric and duodenal juices in order to evaluate the degradation of milk proteins during digestion (Almaas et al., 2011; Furlund et al., 2013). Although those studies have investigated different aspects of β-CN digestion, the presence of potential IgE epitopes in the digestion products has not been addressed. In

this regard, peptide microarray, a technique based on a similar principle than an ELISA protocol, which allows the detection of binding events at an epitope level, could be useful. This technique uses a collection of peptides displayed on a solid surface, usually a glass chip. After being incubated with a variety of serum samples and several washing steps, a fluorescence laballed secondary antibody with the needed specificity is applied. Previous studies have demonstrated that peptide microarray immunoassays constitute a promising technology for epitope determination (Lin et al., 2009).

The main aim of this research was to immunologically characterize the digestion products of β-CN. For this purpose, *in vitro* hydrolysis of β-CN was compared using simulated and human digestive fluids, mimicking three areas of the gastrointestinal tract: the mouth, stomach and small intestine. The reactivity of IgE antibodies towards the digests was assessed, the peptide products obtained were identified by RP-HPLC-MS/MS and the IgE-binding properties of the most relevant peptides resulting in potential epitopes were evaluated by a microarray-based immunoassay. Part of this work was carried out during a 4-month stay at the Mount Sinai School of Medicine in New York.

4.1.1. RESULTS

❖ *4.1.1.1. Digestibility and IgE binding of β-CN*

As shown in Figure 2a, 2-min *in vitro* oral digestion period of β-CN, either with simulated oral fluid or human saliva, did not show any effect on the β-CN pattern as estimated by SDS-PAGE performed using bis-tris 12% acrylamide gels with reducing agents (Figures 2a and 2b, lane 3). Regarding the *in vitro* gastric phase, digestion with

human and simulated fluids also showed similar trends. β-CN was quickly degraded in the early stages of digestion and no residual β-CN was observed by the end of the gastric phase (Figures 2a and 2b). With porcine pepsin, β-CN was broken down into three fragments with molecular masses of, approximately, 23, 18 and 14 kDa, together with fragments of 10 kDa and less. The intact protein and the fragment of 23 kDa disappeared after about 2 and 4 min of digestion, respectively, while the band of 14 kDa resisted for 10 min (Figure 2a, lanes 5, 6 and 9). The concomitant appearance of an additional band of around 12 kDa, together with lower molecular mass peptides was also observed (Figure 2a, lanes 9-12).

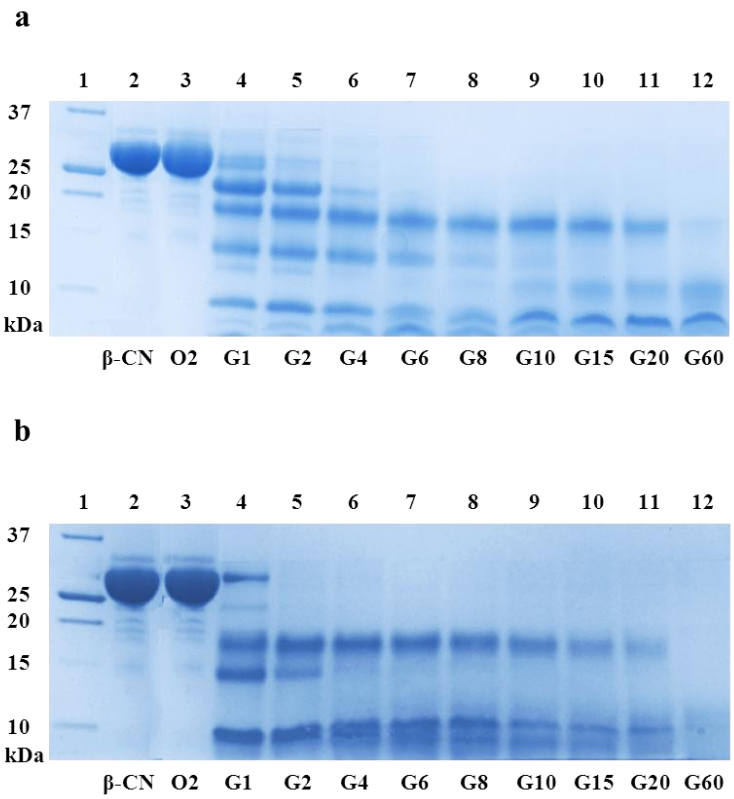

Figure 2. SDS-PAGE patterns, using Bis-Tris 12% acrilamide gels, of β-CN after *in vitro* oral and gastric digestions with simulated (a) and human (b) gastric fluids. Lane 1: molecular mass marker; lane 2: β-CN, lane 3: 2-min oral digests (O2); lanes 4-12: 1 (G1), 2 (G2), 4 (G4), 6 (G6), 8 (G8), 10 (G10), 15 (G15), 20 (G20) and 60-min (G60) gastric digests.

Human fluids yielded basically the same profile, but digestion progressed more rapidly: β-CN was degraded in only two min and the band of 18 kDa, the product most resistant to hydrolysis, was no longer present after 60 min of digestion (Figure 2b, lanes 5 and 12). No bands were detected by SDS-PAGE in the gastroduodenal digests but, as estimated by the number and intensity of the small or hydrophilic peptides with low retention time in the RP-HPLC analyses, hydrolysis with pancreatic enzymes was also more rapid when human fluids were used (not shown).

As shown in Figure 3, reactivity against IgE was evaluated by inhibition ELISA using the sera from 6 milk allergic patients (nos. 1-6, Annex 1). Immunoreactivity of β-CN at the end of gastric digestion increased, as detected by 4 out of the 6 sera, to values reaching 200% of the initial IgE binding of the undigested protein. Despite individual variations, and in agreement with the lowest protein degradation, the highest IgE binding corresponded to the digests produced with commercial porcine pepsin. IgE binding fell drastically at the end of the duodenal phase of the in vitro digestion either with commercial or human enzymes (Figure 3).

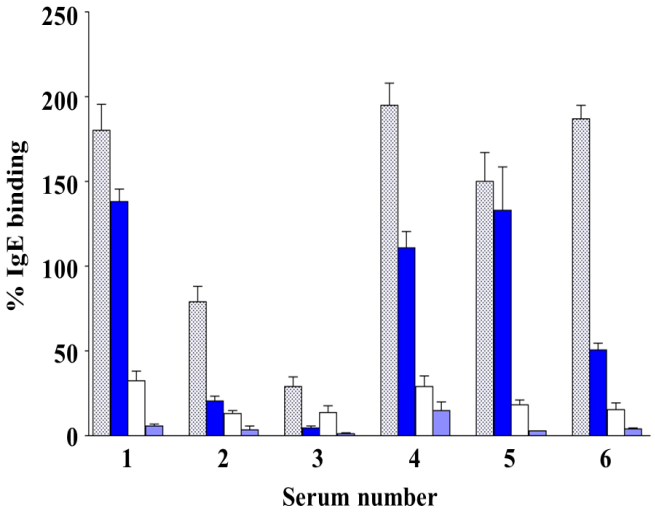

Figure 3. IgE binding of β-CN, expressed as percentage of the EC50 of the intact protein as estimated by inhibition ELISA with sera from six allergic patients (nos. 1-6, Annex 1), after *in vitro* gastric digestion with simulated ( ) and human ( ) fluids and gastroduodenal digestion with simulated ( ) and human ( ) fluids.

Western blotting was used to assess the immunoreactivity against IgE of the degradation products (Figure 4). The different individual sera showed different specificities, but in general terms, the IgE binding of the proteolysis fragments released by pepsin during the first stages of *in vitro* gastric hydrolyses was progressively reduced as the digestions proceeded. However, in the gastric digests with both simulated and human fluids, a band of around 6.5 kDa was recognized, even after 60 min of hydrolysis, by sera from patients 1 and 4, which gave a high response by inhibition ELISA (Figures 3, 4b and 4c), but not by serum from patients 2 (Figure 3 and 4d) and 3 (not shown) which gave a low response. It is, therefore, likely that this degradation product contributed to the residual IgE binding of the in vitro gastric digests, although its actual relevance is probably hampered by an inefficient transfer yield in the electroblotting, as revealed by Ponceau staining of the nitrocellulose membrane (results not shown).

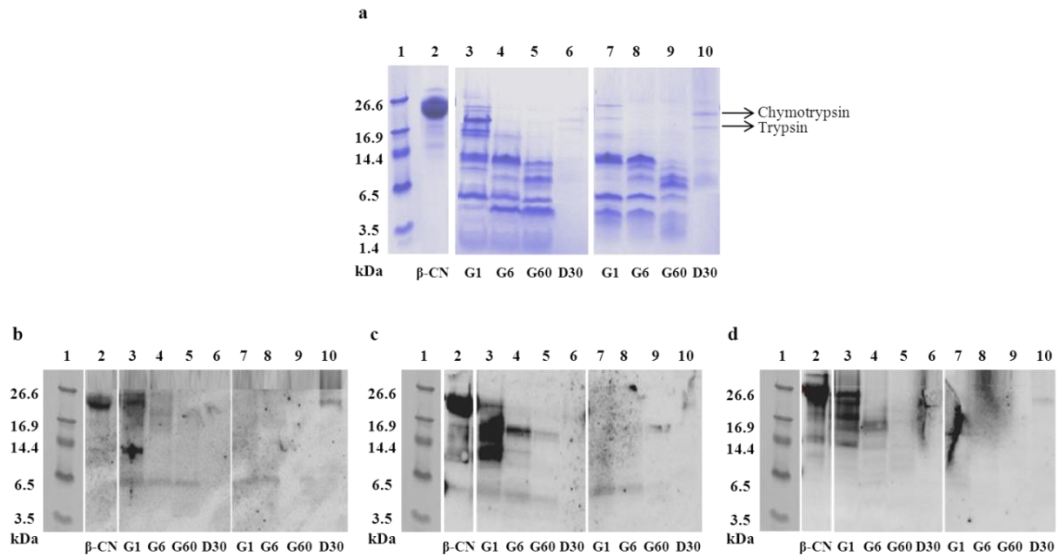

Figure 4. SDS-PAGE patterns (a), using Tris-Tricine 16.5% acrylamide gels, and Western blotting (b, c and d) of β-CN after *in vitro* gastric and gastroduodenal digestions with simulated (lanes 3-6) and human (lanes 7-10) fluids. Lane 2: β-CN; lanes 3-5 and 7-9: 1 (G1), 6 (G6), and 60-min (G60) gastric digests; lane 6 and 10: gastroduodenal digests (60 min of gastric followed by 30 min of duodenal digestion, D30). Sera from patients 1 (b), 4 (c) and 2 (d), described in Annex 1.

❖ *4.1.1.2. Peptide identification after in vitro digestions and epitope recognition*

RP-HPLC-MS/MS was used for the analysis of the gastric (60 min) and gastroduodenal (with a further 30 min of duodenal hydrolysis) digests of β-CN. The sequences of the 152 peptides identified ranged between 3 and 21 amino acids (Figure 5 and Annex 6). The sequence coverage was high: 67.0 and 80.4 % after gastric digestion and 82.8 and 70.3 after subsequent duodenal digestion with simulated and human fluids, respectively. The peptide patterns of the hydrolysates produced in both models were fairly similar following gastric digestion, showing 20 peptides in common, although the human fluids gave rise to a higher proportion of peptides of less than 1,500 Da. Because the range of m/z scanned in RP-HPLC-MS/MS was between 100-3,000 m/z, the peptide mass distribution of the gastric digests was analysed by MALDI-TOF MS, that showed that 17% of the peptides produced after 60 min of gastric digestion were between 3,000 to 10,000 Da (Figure 6). Gastroduodenal digestion with human fluids gave less numerous peptides, all shorter than 1,500 Da (Annex 6).

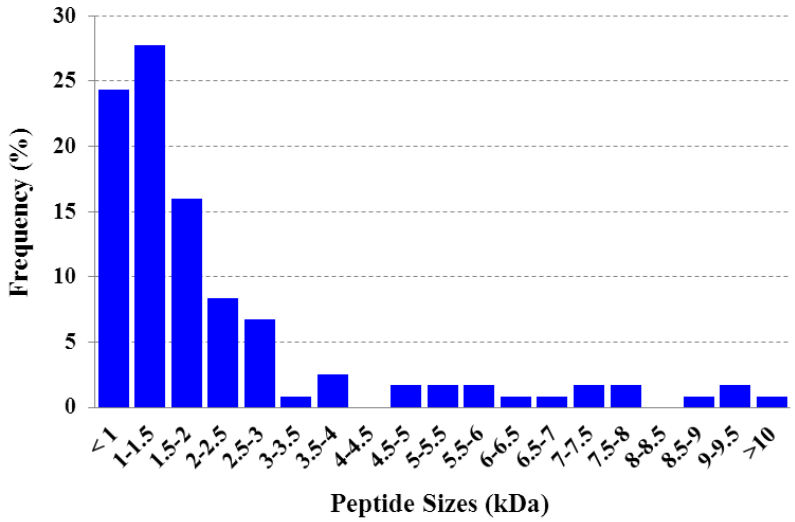

Figure 6. Peptide mass frequency distribution of the gastric digests with simulated fluids shown in a histogram, where each bar corresponds to a peptide size interval of 0.5 kDa.

Among the identified peptides from the duodenal phases in both models of digestion, 12 peptides were selected as is described in materials and methods section. These selected peptides, together with a library of peptides consisting of 20 amino acids overlapping by 17 (3-offset) corresponding to the primary sequence of β-CN, were commercially synthesized to performed peptide microarray. Taking together the 20-amino acid synthetic peptides, the IgE binding areas identified covered almost the whole β-CN sequence, although the area of the protein that was recognized by most sera corresponded to that between residues 31-101 and 124-209 (Figure 5). The IgE-binding patterns of the 12 printed peptides selected from the gastroduodenal digests with simulated and human fluids are also shown in Figure 5, and Table 3 compares their binding frequency to the individual sera with that of matching sequences from the 20- amino acid peptide library. The highest IgE binding (58% of patients) corresponded to the β-CN region comprised between positions 57-93 [β-CN (57-68), β-CN (69-80), β-CN (73-84) and β-CN (82-93)]. Peptides belonging to the region β-CN (144-163) and to the C-terminus [β-CN (191-209)]

showed a moderate IgE binding, being in both cases recognized by approximately 16% of the patients included in the study, while peptides corresponding to the regions between positions 96-126 [β-CN (96-105), β-CN (106-119), β-CN (108-125) and β-CN(114-126)], β-CN (143-156) and β-CN (171-180) did not react with any of the patient's sera employed in the study.

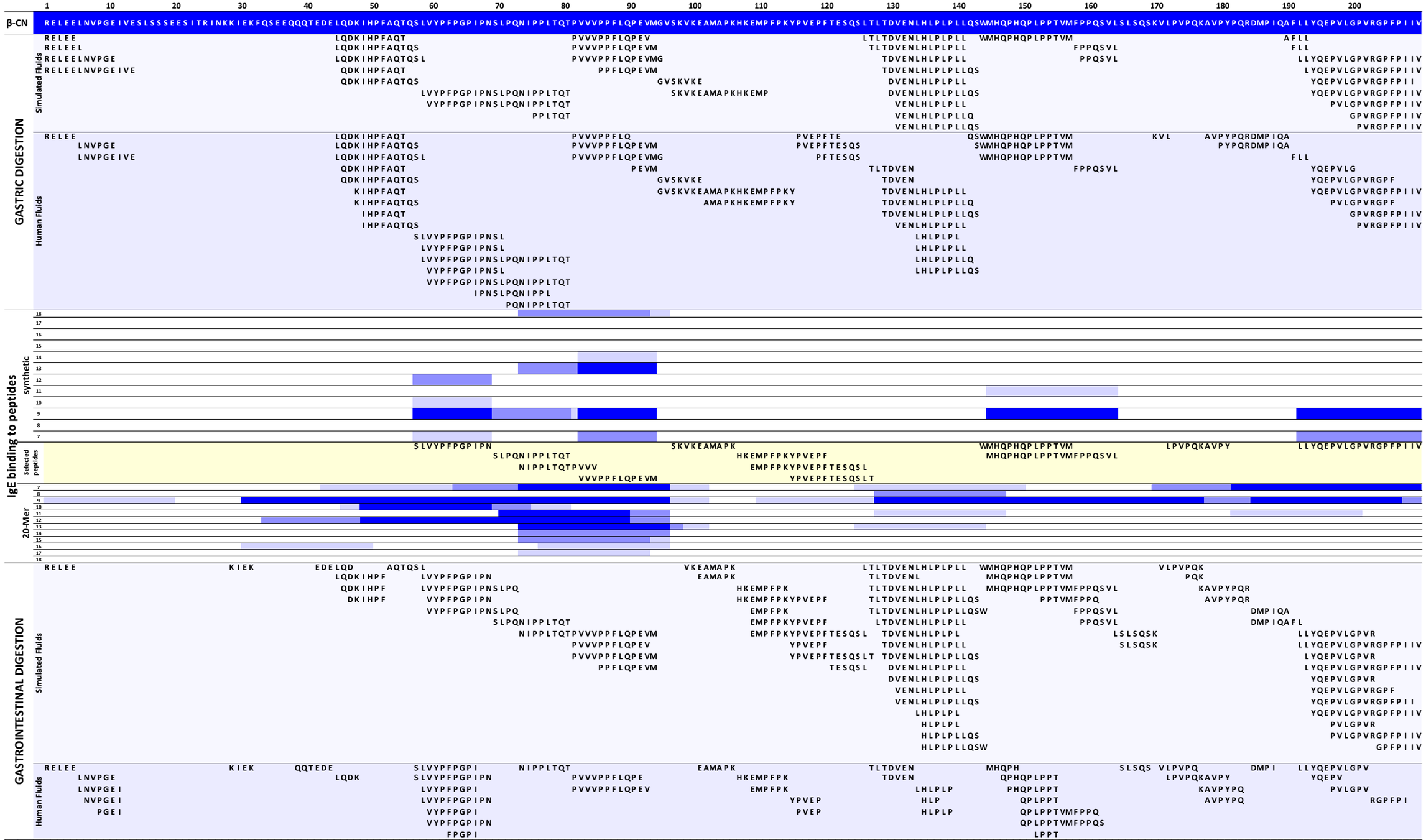

Figure 5. Peptide sequences, identified by RP-HPLC-MS/MS, in the gastric (60 min) and gastroduodenal digests of β-CN (60 min of gastric followed by 30 min of duodenal digestion), with simulated and human fluids, and IgE binding, estimated by peptide microarray with 12 sera of milk allergic patients (patients 7-18, Annex 1), of 12 peptides selected from the simulated digestions and 58 20-amino acids peptides from a peptide library that spans the whole β-CN sequence. The intensity of the IgE binding, low ( ), medium ( ) and high ( ) is shown for each individual serum.

Table 3. IgE-binding (expressed as the number of allergic patients with a positive response towards each peptide) to synthetic peptides derived from *in vitro* digestions of β-CN and related 20-mer peptides from a peptide library that spans the whole β-CN sequence. The matching β-CN fragments identified in the *in vitro* gastric (60 min, G60) and gastroduodenal digests (60 min of gastric followed by 30 min of duodenal digestion, D30) with simulated and human digestive fluids by RP-HPLC-MS/MS are also shown, highlighting in blue the part of the sequence that coincides with the synthetic peptide.

Synthetic peptides derived from digestions	β-CN 20-amino acids peptides	β-CN fragments	Reactive patients	RP-HPLC-MS/MS identified peptides			
				Simulated fluids		Human fluids	
				G60	D30	G60	D30
SLVYPFPGPIPN		57-68	33.3				
	IHPFAQTQ**SLVYPFPGPIPN**	49-68	33.3	**LVYPFPGPIPN**SLPQNIPPLTQT	**LVYPFPGPIPN**	**SLVYPFPGPIPN**SL	**SLVYPFPGPI**
	FAQTQ**SLVYPFPGPIPN**SLP	52-71	33.3	**VYPFPGPIPN**SLPQNIPPLTQT	**LVYPFPGPIPN**SLPQ	**LVYPFPGPIPN**SL	**SLVYPFPGPIPN**
	TQ**SLVYPFPGPIPN**SLPQNI	55-74	33.3		**VYPFPGPIPN**	**LVYPFPGPIPN**SLPQNIPPLTQT	**LVYPFPGPI**
					VYPFPGPIPNSLPQ	**VYPFPGPIPN**SL	**LVYPFPGPIPN**
						VYPFPGPIPNSLPQNIPPLTQT	**VYPFPGPI**
							VYPFPGPIPN
SLPQNIPPLTQT		69-80	8.3				
	PFPGPIPN**SLPQNIPPLTQT**	61-80	33.3	LVYPFPGPIPN**SLPQNIPPLTQT**	**SLPQNIPPLTQT**	LVYPFPGPIPN**SLPQNIPPLTQT**	**NIPPLTQT**
	GPIPN**SLPQNIPPLTQT**PVV	64-83	8.3	VYPFPGPIPN**SLPQNIPPLTQT**		VYPFPGPIPN**SLPQNIPPLTQT**	
	PN**SLPQNIPPLTQT**PVVVPP	67-86	0	**PPLTQT**		IPN**SLPQNIPPL**	
						PQNIPPLTQT	
NIPPLTQTPVVV		73-84	16.7				
	PNSLPQ**NIPPLTQTPVVV**PP	67-86	0	LVYPFPGPIPNSLPQ**NIPPLTQT**	SLPQ**NIPPLTQT**	LVYPFPGPIPNSLPQ**NIPPLTQT**	**NIPPLTQT**
	LPQ**NIPPLTQTPVVV**PPFLQ	70-89	16.7	VYPFPGPIPNSLPQ**NIPPLTQT**	**NIPPLTQTPVVV**PPFLQPEVM	VYPFPGPIPNSLPQ**NIPPLTQT**	**PVVV**PPFLQPE
	NIPPLTQTPVVVPPFLQPEV	73-92	83.3	**PPLTQT**	**PVVV**PPFLQPEV	IPNSLPQ**NIPPL**	**PVVV**PPFLQPEV
				PVVVPPFLQPEV	**PVVV**PPFLQPEVM	PQ**NIPPLTQT**	
				PVVVPPFLQPEVM		**PVVV**PPFLQ	
				PVVVPPFLQPEVMG		**PVVV**PPFLQPEVM	
						PVVVPPFLQPEVMG	
VVVPPFLQPEVM		82-93	41.7				
	PLTQTP**VVVPPFLQPEVM**GV	76-95	83.3	P**VVVPPFLQPEV**	NIPPLTQTP**VVVPPFLQPEVM**	P**VVVPPFLQ**	P**VVVPPFLQPE**
	QTP**VVVPPFLQPEVM**GVSKV	79-98	25	P**VVVPPFLQPEVM**	P**VVVPPFLQPEV**	P**VVVPPFLQPEVM**	P**VVVPPFLQPEV**
	VVVPPFLQPEVMGVSKVKEA	82-101	25	P**VVVPPFLQPEVM**G	P**VVVPPFLQPEVM**	P**VVVPPFLQPEVM**G	
				PPFLQPEVM	**PPFLQPEVM**	**PEVM**	
SKVKEAMAPK		96-105	0				
	EVMGV**SKVKEAMAPK**HKEMP	91-110	0	GV**SKVKE**	**VKEAMAPK**	GV**SKVKE**	**EAMAPK**
				SKVKEAMAPKHKEMP	**EAMAPK**	GV**SKVKEAMAPK**HKEMPFPKY	
						AMAPKHKEMPFPKY	
HKEMPFPKYPVEPF		106-119	0				
	EAMAPK**HKEMPFPKYPVEPF**	100-119	0	SKVKEAMAPK**HKEMP**	**HKEMPFPK**	GVSKVKEAMAPK**HKEMPFPKY**	**HKEMPFPK**
	APK**HKEMPFPKYPVEPF**TES	103-122	0		**HKEMPFPKYPVEPF**	AMAPK**HKEMPFPKY**	**EMPFPK**
	HKEMPFPKYPVEPFTESQSL	106-125	0		**EMPFPK**	**PVEPF**TE	**YPVEP**
					EMPFPKYPVEPF	**PVEPF**TESQS	**PVEP**
					EMPFPKYPVEPFTESQSL		
					YPVEPF		
					YPVEPFTESQSLT		
EMPFPKYPVEPFTESQSL		108-125	8.3				
	HK**EMPFPKYPVEPFTESQSL**	106-125	0	SKVKEAMAPKHK**EMP**	HK**EMPFPK**	GVSKVKEAMAPKHK**EMPFPKY**	HK**EMPFPK**
					HK**EMPFPKYPVEPF**	AMAPKHK**EMPFPKY**	**EMPFPK**
					EMPFPK	**PVEPFTE**	**YPVEP**
					EMPFPKYPVEPF	**PVEPFTESQS**	**PVEP**
					EMPFPKYPVEPFTESQSL	**PFTESQS**	
					YPVEPF		
					YPVEPFTESQSLT		
					TESQSLTL		
YPVEPFTESQSLT		114-126	0				
	MPFPK**YPVEPFTESQSLT**LT	109-128	8.3		HKEMPFPK**YPVEPF**	**PVEPFTE**	**YPVEP**
					EMPFPK**YPVEPF**	**PVEPFTESQS**	**PVEP**
					EMPFPK**YPVEPFTESQSL**	**PFTESQS**	
					YPVEPF		
					YPVEPFTESQSLT		
					TESQSLTL		
WMHQPHQPLPPTVM		143-156	0				
	LLQS**WMHQPHQPLPPTVM**FP	139-158	8.3	**WMHQPHQPLPPTVM**	**WMHQPHQPLPPTVM**	QS**WMHQPHQPLPPTVM**	**MHQPH**
	S**WMHQPHQPLPPTVM**FPPQS	142-161	8.3		**MHQPHQPLPPTVM**	S**WMHQPHQPLPPTVM**	**QPHQPLPPT**
					MHQPHQPLPPTVMFPPQSVL	**WMHQPHQPLPPTVM**	**PHQPLPPT**
					PPTVMFPPQ		**QPLPPT**
					QPLPPTVMFPPQ		**QPLPPTVM**FPPQS
					QPLPPTVMFPPQS		**LPPT**
					LPPT		
MHQPHQPLPPTVMFPPQSVL		144-163	16.7				
	HQPHQPLPPTVMFPPQSVLS	145-164	8.3	W**MHQPHQPLPPTVM**	W**MHQPHQPLPPTVM**	QSW**MHQPHQPLPPTVM**	**MHQPH**
				FPPQSVL	**MHQPHQPLPPTVM**	SW**MHQPHQPLPPTVM**	**QPHQPLPPT**
				PPQSVL	**MHQPHQPLPPTVMFPPQSVL**	W**MHQPHQPLPPTVM**	**PHQPLPPT**
					PPTVMFPPQ	**FPPQSVL**	**QPLPPT**
					FPPQSVL		**QPLPPTVMFPPQ**
					PPQSVL		**QPLPPTVMFPPQS**
							LPPT
LPVPQKAVPY		171-180	0				
	LSLSQSKV**LPVPQKAVPY**PQ	163-182	8.3		V**LPVPQK**	**AVPY**PQRDMPIQA	V**LPVPQ**
	SQSKV**LPVPQKAVPY**PQRDM	166-185	8.3		**PQK**	**PY**PQRDMPIQA	**LPVPQKAVPY**
	KV**LPVPQKAVPY**PQRDMPIQ	169-188	16.7		**KAVPY**PQR		**KAVPY**PQ
					AVPYPQR		**AVPY**PQ
LLYQEPVLGPVRGPFPIIV		191-209	16.7				
	F**LLYQEPVLGPVRGPFPIIV**	190-209	16.7	AF**LL**	**LLYQEPVLGPVR**	F**LL**	**LLYQEPVLGPV**
				F**LL**	**LLYQEPVLGPVRGPFPIIV**	**YQEPVLG**	**YQEPV**
				LLYQEPVLGPVRGPFPIIV	**LYQEPVLGPVR**	**YQEPVLGPVRGPF**	**PVLGPV**
				LYQEPVLGPVRGPFPIIV	**LYQEPVLGPVRGPFPIIV**	**YQEPVLGPVRGPFPIIV**	**RGPFPI**
				YQEPVLGPVRGPFPII	**YQEPVLGPVR**	**PVLGPVRGPF**	
				YQEPVLGPVRGPFPIIV	**YQEPVLGPVRGPF**	**GPVRGPFPIIV**	
				PVLGPVRGPFPIIV	**YQEPVLGPVRGPFPII**	**PVRGPFPIIV**	
				GPVRGPFPIIV	**YQEPVLGPVRGPFPIIV**		
				PVRGPFPIIV	**PVLGPVR**		
					PVLGPVRGPFPIIV		
					GPFPIIV		

4.1.2. DISCUSSION

The results showed that β-CN was unaffected by oral digestion either with simulated oral fluid or human saliva, but quickly degraded afterwards in simulated and human stomach fluids, leaving no intact protein at the end of the gastric phase. This is in accordance with previous *in vitro* studies performed with commercial porcine pepsin in simulated gastric fluid (Dupont et al., 2010a, 2010b). SDS-PAGE analyses revealed a faster degradation of β-CN when digested with human fluids (Figure 2). Even if there is not a consensus on the physiologically representative enzyme to protein ratios that should be used in a digestibility study, both digestion systems were standardized in terms of their proteinase activity on the basis of previously proposed physiologically relevant model (Moreno et al., 2005). Therefore, the variations encountered could be attributed to differences in the specificity and efficiency between the commercial digestive enzymes of porcine (pepsin) and bovine origin (trypsin and α-chymotrypsin) and the human digestive enzymes, as well as to the presence of peptidases, mainly carboxypeptidases (Lyons and Fricker, 2011), in the human pancreatic extracts, which could account for the most extensive *in vitro* duodenal liberation of free amino acids from the peptides previously formed during the gastric phase (Figure 4a and Annex 6). On the other hand, it is known that interaction with physiological surfactants, such as phospholipids and bile salts, plays a key role in protein digestion (Mackie and Macierzanka, 2010). Phosphatidylcholine, a phospholipid secreted by the gastric mucosa, was included in our simulated gastric system, although it has been reported that it does not affect the kinetics of pepsinolysis of β-CN in solution (Macierzanka et al., 2009). Bile acids, that solubilize dietary fats, particularly lipolysis products of triglycerides, through the formation of mixed micelles with phospholipids and fatty acids, also facilitate the proteolysis by pancreatic enzymes

of several dietary proteins, such as β-lactoglobulin (Gass et al., 2007) and ovalbumin (Martos et al., 2010). However, in view of the low concentration of bile acids present in the human duodenal fluid, probably due to prolonged fasting (Kalantzi et al., 2006), it is not likely that it could favour the duodenal proteolysis of the bigger peptides generated from β-CN by pepsin hydrolysis.

There was a high degree of correlation between the digestion products released by simulated and human fluids, particularly after gastric digestion (Figure 5 and Annex 6). The peptides identified by RP-HPLC-MS/MS after pepsin digestion covered most of β-CN sequence, except for the area between the residues 14 and 45, which comprises 5 phophoseryl groups at positions 15, 17, 18, 19 and 35. The resistance of casein phosphopeptides to gastrointestinal digestion has been demonstrated *in vitro* and *in vivo*; therefore, the absence of peptides corresponding to this part of the protein is probably due to the low ionization efficiency of highly electronegative phosphorylated peptides during mass spectrometry analysis (García-Nebot et al., 2010). Following gastroduodenal digestion with simulated fluids, 68 peptides were found, many of which were either totally or partially coincident with fragments previously identified from gastroduodenal hydrolyses of β-CN using *in vitro* systems that mimic adult and an infant models of digestion (Dupont et al., 2010a, 2010b). Gastroduodenal digestion with the human fluids gave 45 peptides, most of which corresponded to fragments shorter than the ones produced by the simulated fluids as a result of a more extensive hydrolysis by the pancreatic extract as discussed above. Therefore, while other studies have shown large differences in the peptide sequences generated from hydrolysis of milk proteins when human and non-human digestive enzymes were compared (Almaas et al., 2011; Furlund et al., 2013), our results suggest that, in view of the similarities between the peptides

released by both systems, the *in vitro* digestion model utilizing commercial enzymes (Moreno et al., 2005) provides a good estimation of the allergenicity of a protein following gastrointestinal digestion.

Accordingly, the IgE binding of the hydrolysates produced with both simulated and human fluids was similar, showing, in 4 out of 6 individual sera used, an increase at the end of the gastric phase, followed by an important decrease when the duodenal digestion was completed, particularly when using human enzymes (Figure 3). The increased IgE-reactivity of β-CN found after the gastric phase, despite the fact that the protein was rapidly degraded upon pepsin digestion suggests that, even if β-CN has a very flexible structure with no disulfide bonds and its major IgE-binding epitopes are assumed to be mainly sequential (Cocco et al., 2007), some IgE-binding epitopes were unmasked following hydrolysis. In fact, caseins constitute a frequently mentioned example of digestion-sensitive proteins that are nonetheless able to sensitize and elicit allergic reactions through the gastrointestinal tract (Wal, 1998). This could be attributed to the formation of IgE-binding protein fragments, such as that with molecular mass of approximately 6.5 kDa, which bound the sera of patients most immunoreactive towards the gastric digests (Figures 4b and c). In addition, MALDI-TOF MS revealed the presence in the gastric digests of peptides with molecular masses between 3,000 and 10,000 Da that could carry IgE epitopes (Figure 6), but might not be detected by Western blotting following SDS-PAGE.

With the development of microarray technology and peptide synthesis techniques, peptide microarray-based immunoassays have proven very useful for epitope mapping of milk allergens (Lin et al., 2009). The use of 58 overlapping peptides showed multiple IgE-binding epitopes distributed throughout the β-CN sequence (Figure 5), with the major

immunoreactive areas, i.e. fragments 31-101 and 124-209, being related with the most hydrophobic parts of the protein: 55-92, 133-150 and 151-202 (Leaver and DaLGleish, 1990). This agrees with the results of previous studies using the peptide microarray technique (Cerecedo et al., 2008; Savilahti et al., 2010) and the SPOTs membrane technique (Chatchatee et al., 2001). Nevertheless, it should be noted that epitope diversity and binding affinity depend heavily on patient selection, especially on the severity of cow´s milk allergy and, in fact, provide prognostic information that may allow distinguishing between transient and persistent food allergy (Wang et al., 2010).

Two IgE-binding synthetic peptides: β-CN (57-68) and β-CN (82-93), related to fragments released from β-CN following *in vitro* digestion with simulated and human fluids, were coincident with the most immunoreactive areas of the protein, in terms of frequency and intensity of IgE binding (Figure 5, Table 3), as well as with sequences previously identified as IgE-binding epitopes, particularly β-CN (57-66), β-CN (52-74) and β-CN (83-92) (Chatchatee et al., 2001; Cerecedo et al., 2008). Dupont et al. (2010a, 2010b) also reported the appearance of β-CN (81-93) after simulated gastrointestinal digestion of β-CN. Six synthetic peptides that resembled products of gastroduodenal digestion, β-CN (96-105), β-CN (106-119), β-CN (108-125), β-CN (114-126), β-CN (143-156) and β-CN (171-180), did not react with any of the patient's sera employed in the study. However, in some cases, IgE-binding was detected when these sequences were included in the 20 amino acid-peptides from the library (see for instance β-CN (143-156) and β-CN (171-180) in Figure 5 and Table 3), thus highlighting the influence of peptide length and the nature of nearby amino acids, in addition to the individual patient specificity, in determining the affinity for IgE antibodies (Cocco et al., 2007; Collis et al., 2003). This raises the question of whether the IgE-binding fragments identified after

gastroduodenal digestion constitute a full epitope or only a part of it. Furthermore, their small molecular mass makes it unfeasible that they contain more than a single IgE binding epitope, suggesting marginal biological activity in terms of basophil activation properties (Albrecht et al., 2009). However, immunization of rats with small peptides, such as those arising from Ara h 1 digestion, induces antibody responses, which could be attributed to their aggregation into complexes of larger sizes (Bøgh, 2012).

Taking into account the limitations inherent in the simulation of *in vivo* gastrointestinal conditions, which are characterised by large inter-individual variations in gastric and duodenal secretions and intra-individual differences due to the type and amount of food consumed or the time of the day (Hur et al., 2011), and the heterogeneous recognition of critical amino acids in β-CN by individual milk allergic patients (Cocco et al., 2007), the similarities found between the *in vitro* simulated digestion system with commercial enzymes used in this work, broadly employed in studies aimed to assess the digestibility of food allergens, and that using human digestive fluids, suggest that the former would provide a reasonably good estimation of the potential allergenicity of protein digests. In fact, by using a peptide microarray-based immunoassay, similar IgE binding epitopes were found within the products of the digested β-CN utilizing both systems, which matched the regions recognized in the peptide library of 58 fragments covering the entire β-CN sequence.

β-LACTOGLOBULIN

4.2. Digestibility and allergenicity of β-Lactoglobulin: simulated gastrointestinal digestion with human and porcine enzymes, characterization, identification and microarray analysis of the hydrolysates

β-Lactoglobulin (β-LG, Bos d 5) is the most abundant whey protein in cow milk, accounting for about 58% of the whey and 10% of the total milk protein. As a member of the lipocalin protein family, β-LG shows a particular tertiary structrure, consisting in a β-barrel with a central calyx with high affinity for hydrophobic ligands. Both characteristics, abundance and a structure endowing an exceptional resistance to digestive enzymes, specially to pepsin (Dalgalarrondo et al. 1995), make it a highly allergenic protein. For instance, bovine β-LG is identified unequivocally as one of the major allergen in cow´s milk, with a reported sensitization rate of 61% (Monaci et al., 2006) although 90% of patients had IgE that recognized β-LG peptide (Wal 1998).

As known, most allergens (including β-LG) are thought to sensitize via the gastrointestinal tract (Bannon, 2004; Mills et al., 2003; Lehrer et al., 1996). Accordingly, there are some studies about digestibility of β-LG. Some of them have reported an enhanced β-LG digestion after heat treatment (80ºC and 1 h) (Chobert et al., 1995), high pressure (Chicón et al. 2008) or emulsification (Macierzanka et al., 2009), and in contrast, others authors have reported reduced digestibility of β-LG by interactions with polysaccharides (Mouecoucou et al., 2007).

Some studies have characterized the resulting products of digestion (Defernez et al., 2010), and others have identified the major IgE binding epitopes (Ball et al., 1994; Wal, 1998; Sélo et al., 1999; Järvinen et al., 2001) but limited work have been reported analyzing the immunoreactive properties of gastrointestinal digests of β-LG, as its high resistance to pepsinolysis makes possible that intact or sufficiently big fragments of β-LG reach the small intestine, which can trigger allergic responses even at low concentrations.

This section described the simulation of the orogastrointestinal proteolysis of β-LG using using human fluids in an *in vitro* digestion system that mimics the successive

passage through the stomach and duodenum and it is compared it with another model that uses commercial enzymes. Then, the identification, IgE-binding properties and epitope mapping of the resulting digestion products have also been evaluated.

4.2.1. RESULTS

❖ *4.2.1.1. Digestibility and IgE binding of β-LG*

The degradation of β-LG, during *in vitro* digestion with human and simulated fluids, was assessed by RP-HPLC and the chromatograms of native β-LG and its digests are shown in Figure 7. The assays were performed at the same enzyme to substrate ratio, taking into account the values described in Materials and Methods section. Native β-LG was eluted at 53 min and although it is hardly cleaved by pepsin throughout gastric digestion, few peptides that eluted at lower retention times in the chromatographic separation than the intact protein were observed, especially in the human fluids digests. Next, in the intestinal environment, β-LG undergoes highly susceptible to proteases, and these hydrophilic peptides increased in abundance as the digestion progressed into the duodenal phase (Fig 7e and 7f). No residual β-LG was observed at the end of the digestion.

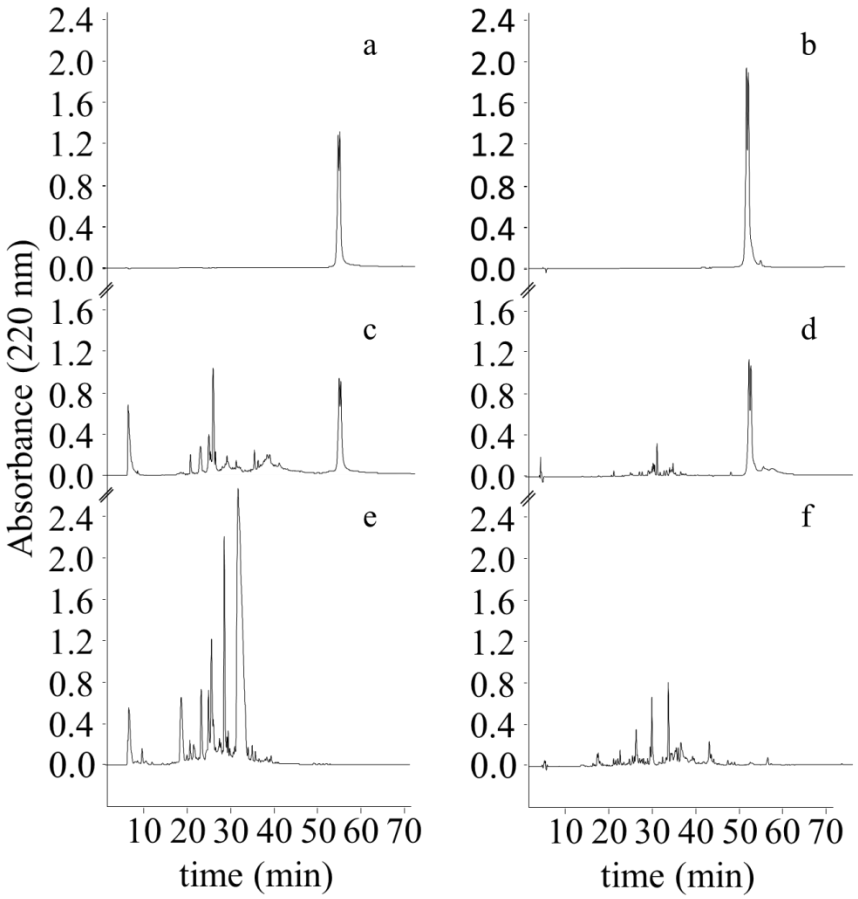

Figure 7. RP-HPLC patterns of β-LG (a, b) subjected to gastric digestión for 60 min (c, d) followed by duodenal digestion for 30 min (e, f) with human (a, c, e) (1.8 mg/mL) and simulated fluids (b, d, f) (3.9 mg/mL).

The human IgE recognition of β-LG before and after *in vitro* gastrointestinal digestions, was tested by inhibition ELISA on the intact protein and on its gastric and duodenal digests, using six different sera from milk allergic patients (nos. 1 to 6, Annex 2). Immunoreactivity of β-LG decreased progressively during digestion, showing values from 40 to 90% of IgE binding in gastric digests when compared to the undigested protein and values close to zero after duodenal digestion (Figure 8). These results agree with the presence of intact protein at the end of the gastric digestion and with the

complete degradation at the end of the duodenal stage observed with both models of digestion studied.

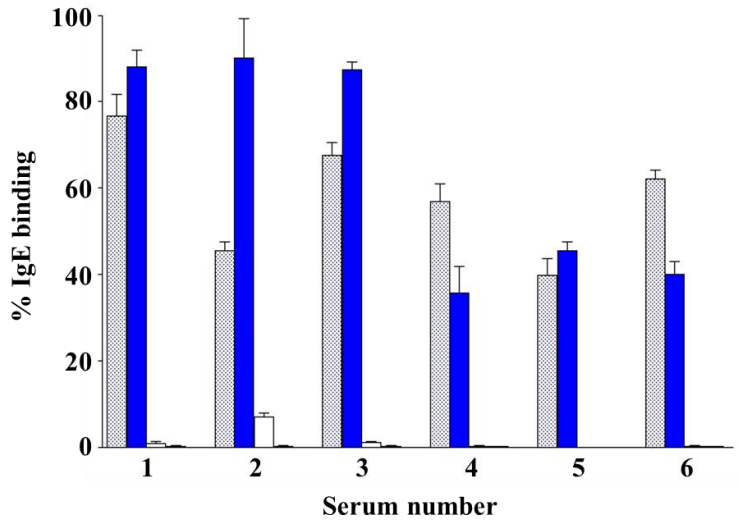

Figure 8. IgE binding of β-LG, expressed as percentage of the EC50 of the intact protein as estimated by inhibition ELISA with sera from six allergic patients (patients 1-6, Annex 2), after *in vitro* gastric digestion with simulated (▒) and human (■) fluids and gastroduodenal digestion with simulated (□) and human (■) fluids.

❖ *4.2.1.2. Peptide identification after in vitro digestions*

The gastric (60 min) and gastroduodenal (with a further 30 min of duodenal hydrolysis) digests of β-LG were subjected to RP-HPLC in tandem with mass spectrometry using a quadrupole ion trap. Figure 9 and Annex 7 show the sequences of the 195 peptides identified, which ranged between 3 and 26 amino acids. The sequence coverage was high: 90.8 and 72.4% after gastric digestion and 96.3 and 74.8% after subsequent duodenal digestion with simulated and human fluids, respectively. The identification of peptides in samples of β-LG digested with human fluids showed 37

peptides in the gastric phase and 57 in the duodenal phase, while simulated fluids produced 75 peptides in the gastric phase and 100 in the duodenal stage. Regarding to the peptides generated in the gastric phase, 21 peptides were coincident in both models of digestion and the most abundant cleavage site in both systems of digestion was leucine at the C-terminal position, showing this pattern the 33.3% of the peptides identified in the digestion carried with simulated fluids and the 29.7% of the peptides identified in the digestion with human fluids. Most of the remaining peptides found in the duodenal digests produced with both models of digestion could be products of hydrolysis of the intact protein remaining after the gastric phase by duodenal enzymes action as it is shown by the breakdown at the trypsin cleavage sites: Lys (8, 14, 60, 69, 75, 83, 91, 100, 135, 138 and 141) and Arg (40, 124 and 148). In the duodenal digest produced with human fluids, 18 peptides were coincident with those produced with simulated fluids.

❖ *4.2.1.3. Peptide microarray immunoassay: Epitope recognition*

22 peptides, ranging from 10 to 19 amino acids, representative of those found in the gastroduodenal digests with simulated and human fluids as well as a library of 36 peptides covering the whole sequence of β-LG were chemically synthetized and their IgE-binding to 8 individual sera from milk allergic patients (nos. 7-14, Annex 2) was assessed by peptide microarray (Figure 9). Taking together the 20-amino acids synthetic peptides, the IgE binding areas identified with the peptide microarray were two, β-LG (21-74) and β-LG (100-149), although the areas of the protein that were recognized by most sera corresponded to that between residues β-LG (43-65) and β-LG (124-140) (Figure 9). These immunodominant reactive areas were coincident with the IgE-binding patterns of

the 22 printed peptides selected from the gastroduodenal digests with simulated and human fluids. Moreover, the area β-LG (86-99) was recognized by 50% of the patients used in this study, while any of the sera showed IgE binding against the same area when it was included in bigger peptides of 20 aminoacids. Peptides from both models of digestion are included in the three regions of the sequence with IgE binding.

Table 4 compares the IgE binding frequency to the individual sera of the selected peptides from digestions with that of matching sequences from the 20-amino acids peptide library. The highest IgE binding (87.5% of patients) corresponded to the β-LG region comprised between positions 43-62 [β-LG (43-60), and β-LG (47-62)]. The peptide β-LG (135-147) was also very immunoreactive, reacting with the 62.5% of the patients. Between 12,5 to 50% of the sera´s patients reacted with peptides β-LG (86-99), β-LG (86-100), β-LG (108-125) β-LG (122-136) and β-LG (123-137), while peptides β-LG (21-32), β-LG (25-35), β-LG (29-41), β-LG (56-69), β-LG (62-80), β-LG (71-82), β-LG (74-88), β-LG (91-105), β-LG (98-111), β-LG (101-115), β-LG (107-116), β-LG (112-126), β-LG (115-129) and β-LG (134-146) did not react with any of the patient's sera employed in the study.

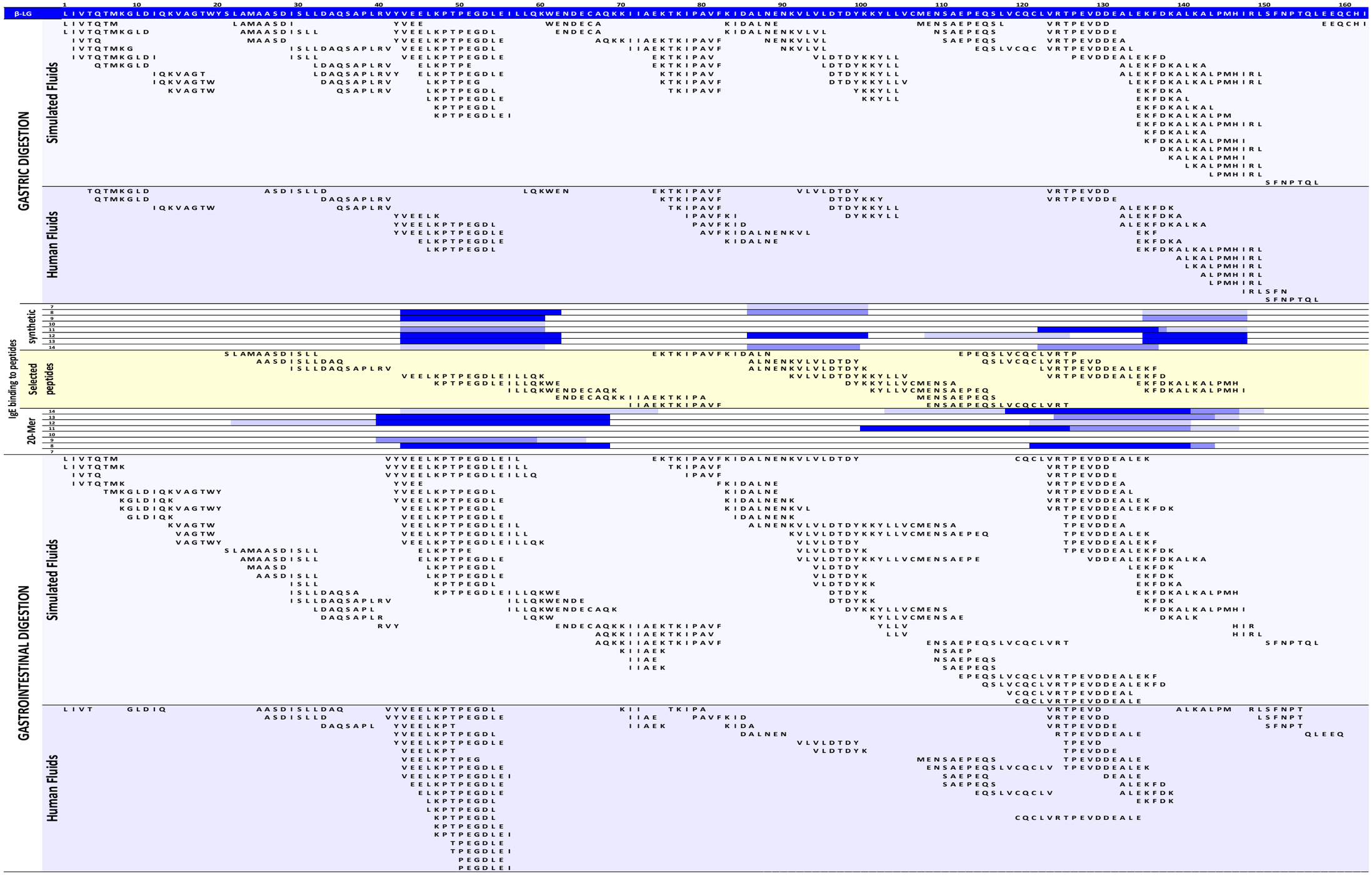

Figure 9. Peptide sequences, identified by RP-HPLC-MS/MS, in the gastric (60 min) and gastroduodenal digests of β-LG (60 min of gastric followed by 30 min of duodenal digestion), with simulated and human fluids, and IgE binding, estimated by peptide microarray with 8 sera of milk allergic patients (patients 7-14, Annex 2), of 22 peptides selected from the simulated digestions and 36 20-amino acid peptides from a peptide library that spans the whole β-LG sequence. The intensity of the IgE binding, low ( ), medium ( ) and high ( ) is shown for each individual serum.

Table 4. IgE-binding (expressed as the percentage of allergic patients with a positive response towards each peptide) to synthetic peptides derived from in vitro digestions of β-LG and related 20-amino acids peptides from a peptide library that spans the whole β-LG sequence. Highlighting in blue the part of the sequence that coincides with the synthetic peptide derived from digestions.

Synthetic peptides derived from digestions	β-LG 20-amino acids peptides	β-LG fragments	% Reactive patients
SLAMAASDISLL		21-32	0
	QKVAGTWY**SLAMAASDISLL**	13-32	0
	AGTWY**SLAMAASDISLL**DAQ	16.35	0
	WY**SLAMAASDISLL**DAQSAP	19-38	0
AASDISLLDAQ		25-35	0
	AGTWYSLAM**AASDISLLDAQ**	16-35	0
	WYSLAM**AASDISLLDAQ**SAP	19-38	0
	LAM**AASDISLLDAQ**SAPLRV	22-41	12.5
ISLLDAQSAPLRV		29-41	0
	LAMAASD**ISLLDAQSAPLRV**	22-41	12.5
	AASD**ISLLDAQSAPLRV**YVE	25-44	0
	D**ISLLDAQSAPLRV**YVEELK	28-47	0
VEELKPTPEGDLEILLQK		43-60	87.5
	VEELKPTPEGDLEILLQKWE	43-62	50
KPTPEGDLEILLQKWE		47-62	37.5
	VEEL**KPTPEGDLEILLQKWE**	43-62	62.5
	L**KPTPEGDLEILLQKWE**NDE	46-65	50
ILLQKWENGECAQK		56-69	0
	GDLE**ILLQKWENDECAQK**KI	52-71	0
	E**ILLQKWENDECAQK**KIIAE	55-74	12.5
ENDECAQKKIIAEKTKIPA		62-80	0
IIAEKTKIPAVF		71-82	0
	DECAQKK**IIAEKTKIPAVF**K	64-83	0
EKTKIPAVFKIDALN		74-88	0
ALNENKVLVLDTDY		86-99	37.5
ALNENKVLVLDTDYK		86-100	50
KVLVLDTDYKKYLLV		91-105	0
	KVLVLDTDYKKYLLVCMENS	91-110	0
DYKKYLLVCMENSA		98-111	0
	T**DYKKYLLVCMENSA**EPEQS	96-116	0
KYLLVCMENSAEPEQ		101-115	0
	K**KYLLVCMENSAEPEQ**SLVC	100-119	12.5
MENSAEPEQS		107-116	0
	KKYLLVC**MENSAEPEQS**LVC	100-119	12.5
	LLVC**MENSAEPEQ**SLVCQCL	103-122	25
	C**MENSAEPEQS**LVCQCLVRT	106-125	25
ENSAEPEQSLVCQCLVRT		108-125	12.5
	CM**ENSAEPEQSLVCQCLVRT**	106-125	25
EPEQSLACQCLVRTP		112-126	0
	NSA**EPEQSLVCQCLVRTP**EV	109-128	0
QSLVCQCLVRTPEVD		115-129	0
	QSLVCQCLVRTPEVDDEALE	115-134	12.5
LVRTPEVDDEALEKF		122-136	25
	VCQC**LVRTPEVDDEALEKF**D	118-137	25
	C**LVRTPEVDDEALEKF**DKAL	121-140	50
VRTPEVDDEALEKFD		123-137	25
	VCQCL**VRTPEVDDEALEKFD**	118-137	25
	CL**VRTPEVDDEALEKFD**KAL	121-140	50
EKFDKALKALPMH		134-146	0
	EVDDEAL**EKFDKALKALPMH**	127-146	37.5
	DEAL**EKFDKALKALPMH**IRL	130-149	12.5
	L**EKFDKALKALPMH**IRLSFN	133-152	0
KFDKALKALPMHI		135-147	62.5
	DEALEKF**DKALKALPMHI**RL	130-149	12.5
	LEKF**DKALKALPMHI**RLSFN	133-152	0

4.2.2. DISCUSSION

The results of RP-HPLC analysis of the hydrolysates from the two models of digestion showed the same pattern of breakdown showing human fluids a greater reaction rate based on the appearance of higher number of peaks during gastric digestion and higher degradation of intact β-LG, although both types of systems left the main part of β-LG undigested at the end of this step (Figure 7). After duodenal phase with both *in vitro* models, β-LG was completely digested. Earlier digestion studies with simulated fluids showed similar results for both gastric and duodenal phases (Schmidt et al 1995; Fu et al. 2002; Chicón et al., 2008). Our results are also consistent for the gastric phase in other previously reported studies with human fluids (Almaas et al. 2006a; Inglingstad et al. 2010), but some differences were found after the duodenal phase. These authors reported about 83 and 60% respectively of intact β-LG in bovine milk at the end of the gastrointestinal digestion. This discrepancy between the results can be due to the different substrate used for the digestion. Minerals, carbohydrate content or buffer capacity of milk could have an impact on the enzymatic activity of human fluids. Moreover the presence of bile salts in the digestion also affects the proteolytic activity of the enzymes. Gass et al. (2007) demonstrated that the addition of bile acid mixture significantly enhanced the digestion of β-LG by trypsin and chymotrypsin. However, in view of the low concentration of bile acids present in the human duodenal fluid, probably due to prolonged fasting (Kalantzi et al., 2006), it is not likely that it could favour the duodenal proteolysis of β-LG.

On the other hand, the lower number and less size of peptides identified in the duodenal stage of the digestion with human fluids compared with those from digestion

with simulated fluids could indicates a higher degradation during digestion using human fluids. This fact is in agreement with our previous findings in β-CN digestion (section 4.1) but against the results obtained using caprine milk and whey by Eriksen et al. (2010) and Almaas et al. (2006b) respectively. However these differences are probably due to the higher ratio of enzyme to substrate used in our study.

As mentioned above, both models of digestion have been carried out with the same ratio enzyme activity-substrate and the large number of different peptides obtained with simulated fluids digestion model and the differences in the cleavages of the peptides could be due to a species difference, with a higher specificity of porcine enzymes. These species-dependent results have also been observed in the hydrolysis of β-casein by human pepsin and bovine chymotrypsin (Guillou et al., 1991). Moreover different isoforms of pepsin (Dunn, 2002) and pancreatic enzymes (Scheele et al., 1981) present in the human fluids may be more efficient at degrading the β-LG than porcine enzymes in the simulated fluids model.

Assessing the digestibility of a protein is not by itself a sufficient measure of its allergenic potential and hence we also have investigated the IgE binding capacity of the digests from both models of digestion. Immunoassays showed in general the same results with both models of digestion (Figure 8). The residual IgE binding capacity of β-LG after two stages of digestion was found to be entirely consistent with the levels of residual native protein found in the digests of the two models with the immunoreactivity of β-LG decreasing progressively after gastrointestinal digestion, due to the disruption of epitopes sites present in the gastric phase. In contrast, Bossios et al. (2011) observed that digestion did not modify the IgE binding capacity of β-LG may be due to differences in the protocol of digestion used. B-LG has both linear and conformational epitopes and during

digestion a reduction in the binding strength between antibodies and antigens is expected, because of the reduced possibility of small peptides to sustain sufficient structural integrity to bind antibodies raised against conformational epitopes (Bøgh et al., 2012).

Previous studies have identified some regions of the protein as epitopes. According to Adams et al. (1991), the main β-LG epitope corresponds to β-LG (124-134), while Ball et al. (1994) posteriorly reported the sequences β-LG (95-104), β-LG (96-105) and β-LG (97-108) as the most important fragment with IgE-binding capacity. Sélo et al. (1999) considered the tryptic peptides β-LG (41-60), β-LG (102-124) and β-LG (149-162) as the major epitopes within β-LG, although other fragments also showed IgE binding capacity, such as β-LG (1-8) and β-LG (25-40). The sequences β-LG (12-27), β-LG (95-113) and β-LG (124-135) were also characterized as IgE epitopes by Heinzmann et al. (1999). Järvinen et al. (2001) identified seven major epitopes of β-LG corresponding to β-LG (1-16), β-LG (31-48), β-LG (47-60), β-LG (67-78), β-LG (75-86), β-LG (127-144) and β-LG (141-152). Epitopes from the region β-LG (1-48), described previously by Sélo et al. (1999), Heinzmann et al. (1999) and Järvinen et al. (2001), β-LG (95-113) (Ball et al., 1994; Heinzmann et al., 1999) and β-LG (141-152) (Järvinen et al., 2001) could be irrelevant when we study the immunoreactivity of the digests because the experimental data reported in this study show that the identified peptides from the gastroduodenal digestion containing in these regions are mostly from the model using simulated fluids and not from the human model. In contrast, the identified peptides from the gastroduodenal digestion containing in the region β-LG (149-162), reported by Sélo et al. (1999) as an epitope, are mainly from the digestion with human fluids. The peptides from gastroduodenal digestion contained in the previously described areas of β-LG as epitopes corresponding to β-LG (41-60) (Sélo et al., 1999; Järvinen et al., 2001), β-LG

(67-86) (Järvinen et al., 2001) and β-LG (102-144) (Adams et al., 1991; Sélo et al., 1999; Heinzmann et al., 1999; Järvinen et al., 2001) were identified in the digests of both human and simulated digestion models and could explain the residual immunogenicity detected by inhibition ELISA at the end of gastroduodenal digestions. Moreover, the fragment β-LG (75-86) has been reported as an important epitope only recognized by the patients with persistent cow´s milk allergy (Järvinen et al., 2001).

With the development of microarray technology and evolution in peptide synthesis techniques, peptide microarray-based immunoassay has been developed for epitope mapping of milk allergens (Lin et al., 2009). In this study, we have identified 3 areas of epitopes of β-LG (Figure 9). Two of these areas have been previously identified as epitopes by microarray technology (Cerecedo et al., 2008; Wang et al., 2010). However, one of the reactive areas in this study (ALNENKVLVLDTDYK) was not identified in previous studies. This new area is only reactive if it corresponds to a complete peptide from digestion but it is not reactive if the sequence is included in peptides of 20 amino acids from the library, showing that in microarray technique the peptide generated during the digestion might have different behaviour than the same peptide included in a bigger sequence. The new region identified includes peptides from both models of digestion. This highlights the influence of peptide length and the nature of nearby amino acids, in addition to the individual patient specificity, in determining the affinity for IgE antibodies (Cocco et al., 2007; Collis et al., 2003). This raises the question of whether the IgE-binding fragments identified after gastroduodenal digestion constitute a full epitope or only a part of it.

In conclusion, the similarities found between the *in vitro* simulated digestion system with simulated fluids used in this work, broadly employed in studies aimed to

assess the digestibility of food allergens, and that using human digestive fluids, suggest that the former would provide a reasonably good estimation of the potential allergenicity of protein digests, although specific epitopes could be preferentially formed by either model. Additional studies are necessary to confirm these findings but peptide microarrays might be a useful technique to complete the research of digestibility and allergenicity of food allergens. Moreover, in order to study the allergenicity of hydrolysates by microarray technique maybe would be better test the real peptides and not an overlapping set of peptides, because the IgE binding can change depending on length of the peptides.

OVALBUMIN

4.3. Identification of IgE binding peptides in hen egg ovalbumin digested *in vitro* with human and simulated gastroduodenal fluids

Ovalbumin (OVA, Gal d 2), the most abundant protein in egg white (up to 54% of the protein content), is considered one of the dominant allergens (Mine and Zhang, 2008). OVA is a phosphoglycoprotein with a molecular mass of 45 kDa and 385 amino acids (McReynolds et al., 1978; Nisbet et al., 1981). X-ray crystallography studies have shown a three dimensional structure consisting of three layers of α-helices and nine β-sheets (Stein et al., 1990) (Figure 10a). OVA sequence includes six cysteines, with a single disulphide bond between Cys_{73} and Cys_{120}, a glycosylation site at Asn_{292}, and two phosphorylation sites at Ser_{68} and Ser_{344}. Half the amino acid residues are hydrophobic (Figure 10b) and one third are charged residues (Figure 10c).

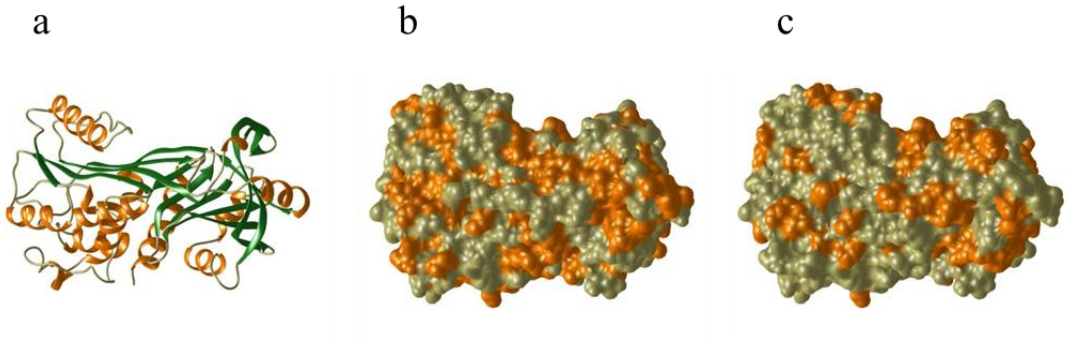

Figure 10. Representation of (a) the secondary structure (α-helix in orange color, β-strand in green and random coil in light brown), (b) hydrophobic amino acid residues (orange color) and (c) charged amino acid residues (orange color) of OVA in the 3D structure of the protein. Molecular graphics and analyses were performed with the UCSF Chimera package (Pettersen et al., 2004). The X-ray coordinates (Stein et al., 1991) were obtained at the RCSB Protein Data Bank (PDB ID: 1OVA).

As many other allergenic proteins, OVA partially resists hydrolysis with pepsin and pancreatic enzymes, an aspect that could contribute to its ability to sensitize or elicit

allergic reactions (Ruiter and Shreffler, 2012). So far, the studies investigating the gastrointestinal stability of OVA have been performed *in vitro* by the use of enzymes of bovine or porcine origin as part of different digestion models, ranging from simple one-step hydrolyses (Fu et al., 2002; Takagi et al., 2003), to advanced physiologically relevant systems where subsequent gastric and duodenal digestions are conducted under conditions that mimic the *in vivo* processes in infants and adults (Dupont et al., 2010a; Martos et al., 2010). However, to the best of our knowledge, the digestibility with human digestive juices has not been assessed.

In the present section, the digestibility of OVA using human gastric and duodenal juices was assessed, the peptides released were characterized and the IgE-binding properties of the most relevant digestion products were evaluated. Digestions were also conducted with simulated fluids for comparative purposes. This allowed the identification of relevant IgE binding epitopes that resisted digestion in both systems and could contribute to the allergenic potential of the protein.

4.3.1. RESULTS

❖ *4.3.1.1. In vitro digestion of ovalbumin with human and simulated fluids*

Figure 11 shows the SDS-PAGE patterns (Figure 11a and 11c) and Western blots using a pool of sera from 6 allergic patients (nos. 7-12, Annex 3) (Figure 11b and 11d) of OVA after being subjected to two-step digestion protocols, mimicking the stomach and duodenal stages, by using human and simulated fluids. In general terms, after 60 min of incubation with human gastric fluid a remarkable amount of OVA still remained

undigested, while the intact protein virtually disappeared following the subsequent duodenal phase (Figure 11a).

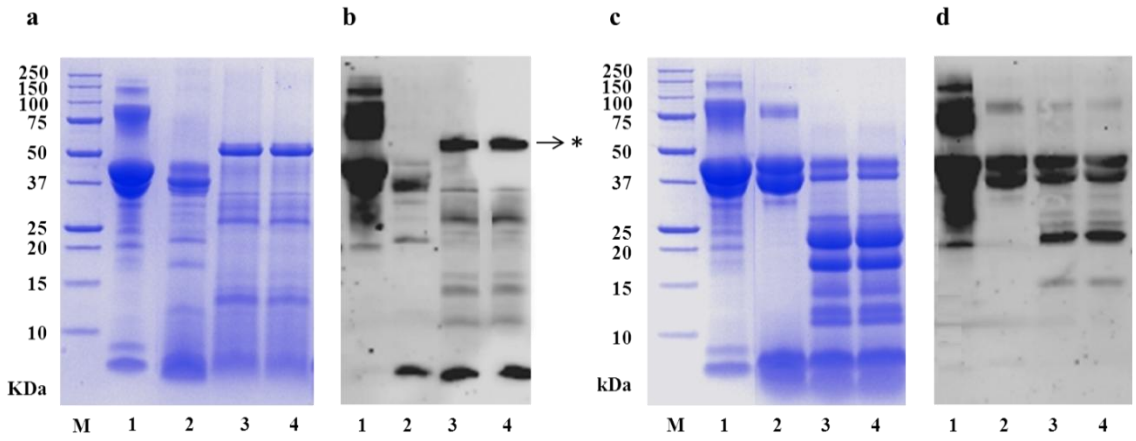

Figure 11. SDS-PAGE (a, c) using Bis-Tris 12% acrylamide gels, and Western blotting (b, d) of OVA after *in vitro* gastric and gastroduodenal digestion with human (a, b) and simulated (c, d) fluids. M: molecular mass marker, lane 1: OVA, lane 2: 60-min gastric digests; lane 3: gastroduodenal digest (60 min of gastric digestion followed by 30 min of duodenal digestion); lane 4: 60 min of gastric digestion followed by 60 min of duodenal digestion). * IgE-binding fragment originally present in the human duodenal fluid.

Gastric and, particularly, duodenal hydrolyses were much faster with human fluids as compared with simulated fluids, with the appearance of more degradation products after the gastric phase, of ~40, 22, 18, 13 and 5 kDa, and less degradation products, within the range of ~25 to 5 kDa, after the duodenal phase (Figure 11a and 11c). As a result, there were less IgE-binding from intact OVA and its 40 kDa fragment following gastroduodenal digestion with human fluids. Remarkably, a strong IgE-binding band of ~21 kDa, that formed during duodenal digestion with simulated fluid, was absent from the corresponding digests produced with human fluid, which in turn, yielded lower molecular mass immunoreactive degradation products (Figure 11b and 11d). It should be

mentioned that no differences in the degradation patterns were detected when comparing 30 and 60 min of duodenal digestion with either system.

In consonance with these results, inhibition ELISA (Figure 12) showed that the gastric and gastroduodenal digests produced with human fluids exhibited the lowest IgE-binding to 4 individual sera (nos. 1, 2, 4 and 5, in Annex 3). With other 2 sera (nos. 3 and 6, Annex 3), IgE-reactivity towards the gastric digests produced by both systems was very similar, although the lowest IgE-binding always corresponded to the human gastroduodenal digests.

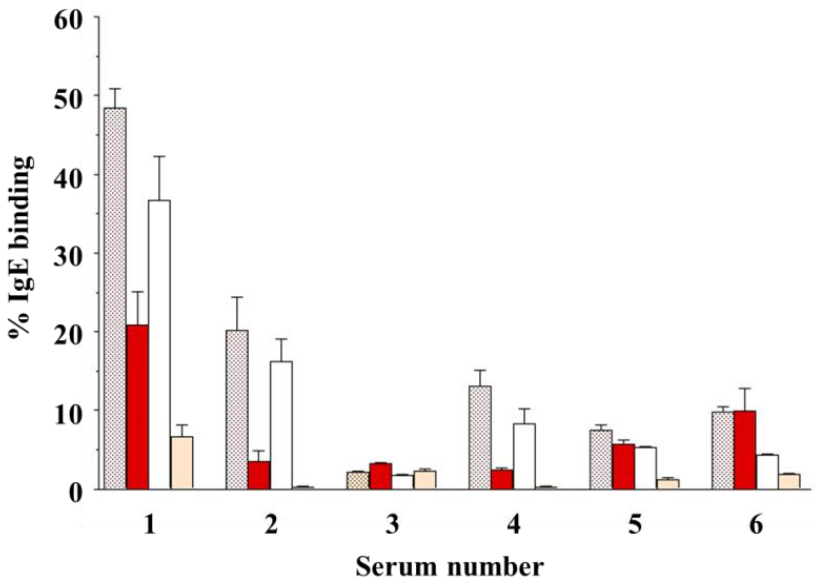

Figure 12. IgE binding of OVA, expressed as percentage of the EC_{50} of the intact protein as estimated by inhibition ELISA with sera from six allergic patients (nos. 1-6, Annex3), after *in vitro* gastric digestion with simulated ( ) and human ( ) fluids and gastroduodenal digestion with simulated ( ) and human ( ) fluids.

❖ *4.3.1.2. Identification of IgE-binding epitopes*

Figure 13 and Annex 8 show the sequences of the peptides identified by RP-HPLC-MS/MS in the gastric (60 min) and gastroduodenal (60 min of gastric digestion followed by 30 min of duodenal digestion) digests of OVA. No peptides covering the first 50 amino acids of the protein sequence were found in either model. Following gastric digestion with human and simulated fluids, 84 and 118 peptides were identified respectively, with 47 peptides in common. There were 52 identical pepsin cleavage sites in both systems, including: Leu (83, 105, 114, 124, 183, 216, 232, 242, 252, 255, 282, 312, 321 and 365), Glu (70, 108, 143, 202, 248, 266, 274, 336, 340 and 357), Phe (59, 99, 134, 198, 217 and 261), Ala (141, 171, 177, 187, 195. 332 and 345), Met (210, 211, 222, 239 and 241) and Gln (89, 140, 203 and 325). 81 out of the 118 sequences identified after gastric hydrolysis with simulated fluid resisted subsequent duodenal hydrolysis, appearing as end products of digestion. Most of the remaining peptides found in the duodenal digests produced with simulated fluid could be products of hydrolysis of the low molecular mass peptides previously released by pepsin action, except for the C-terminal sequence HIATNAVLFFGRCVS, resulting from the trypsin cleavage at Lys369. In addition, in this digestion system, breakdown at the trypsin cleavage sites: Lys (55, 92, 226, 228, 277, 279, 286 and 322) and Arg (50, 84, 104, 142, 158 and 276) was detected. Only 47 peptides were identified after gastrointestinal digestion with human fluids. In this case, there were several fragments which could not arise from the peptides previously identified after gastric digestion and thus, they could derive from the hydrolysis of either intact OVA or the big proteolysis fragments that resisted pepsin hydrolysis. Only 6 peptides were coincident with those produced with simulated duodenal fluid and, in

general terms, the human fluid gave rise less numerous and shorter peptides. Hydrolysis by trypsin at Lys 290 and Arg (50, 158, 284 and 359) was detected (Figure 13 and Annex 8).

27 peptides, ranging from 10 to 16 amino acids, representative of those found in the gastroduodenal digests with simulated and human fluids were chemically synthetized and their IgE-binding to 10 individual sera from egg-allergic patients was assessed by dot-blot. As shown in Figure 13, all peptides were recognized by, at least, 1 serum, except for two fragments, OVA (299-308) and (351-360), which did not react with any of the patient's sera employed in the study. A high IgE-binding corresponded to the region comprised between positions 125-176, because 40 to 60% of the patients reacted to the synthetic peptides OVA (125-134), OVA (135-144), OVA (141-154), OVA (159-172) and OVA (164-176). The fragments OVA (188-198) and OVA (326-336) were recognised by sera from 60 and 50% of the patients respectively. The highest IgE-binding corresponded to the C-terminal fragment OVA (370-385), which reacted with 80% of the sera.

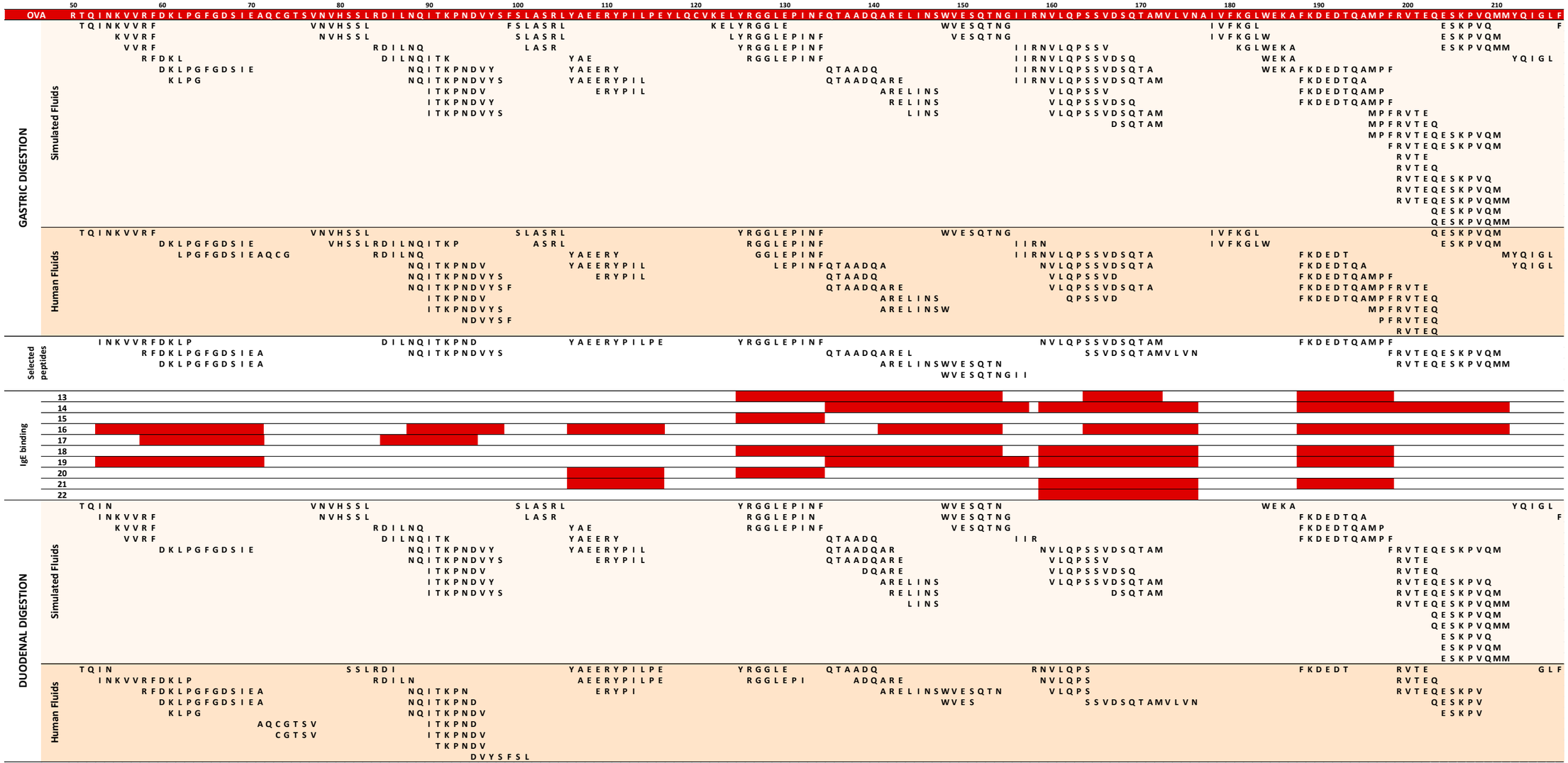

Figure 13. Peptide sequences, identified by RP-HPLC-MS/MS, in the gastric (60 min) and subsequent duodenal digests of OVA (60 min of gastric digestion followed by 30 min of duodenal digestion), and IgE binding (■), estimated by dot-blot with 10 sera of egg-allergic patients (patients 13-22, Annex 3), of 27 synthetic peptides selected within the peptides identified in the simulated digestions.

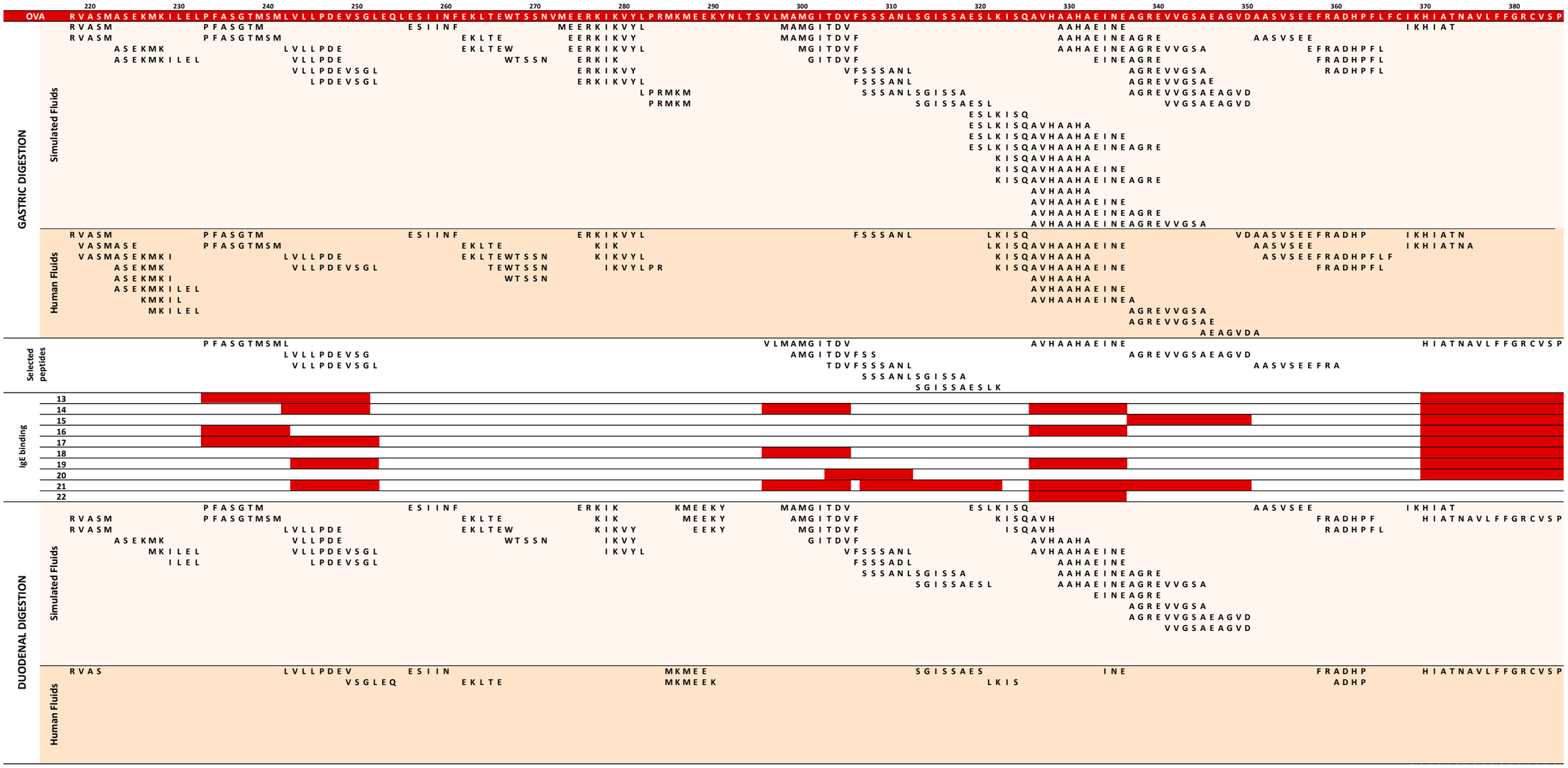

Figure 4. Continuation.

4.3.2. DISCUSSION

The results obtained with simulated gastric fluid agree with previously published information showing that OVA is quite stable to porcine pepsin at an E:S ratio of 182 U/mg and pH 2.0 (Martos et al., 2010). A digestion pattern with two main degradation products, of 40.1 and 4.1 kDa, has been described (Dearman at al., 2002; Takagi et al., 2003; Thomas et al., 2004), the first one probably corresponding to the fragment Ala23-Pro385 (Martos et al., 2010). Moreover, Dupont et al. (2010a) using a gastric model under similar conditions (E:S 182 U/mg, pH 2.5) reported, in addition to smaller peptides of ~5,400 and 4,600 Da, the appearance of digestion products of ~21,700 and 17,800 Da, which are analogous to those found in the gastric digests produced with human fluid in the present study (Figure 11a). In any case, and though there was intact protein remaining after the gastric phase in both systems, degradation of OVA was faster when digested with human than with simulated fluid (Figure 11a and 11c), an aspect that was already observed with β-CN and β-LG in sections 4.1 and 4.2. This suggests a more efficient performance of pepsin of human origin despite specificity was similar, as judged by the existence of 52 identical cleavage sites and a similar peptide pattern with 47 peptides in common (Figure 13 and Annex8. It is noteworthy that the *in vitro* digestion with human gastric juice released peptides with antihypertensive activity that act either as inhibitors of angiotensin-I-converting enzyme or exert a vasodilator action, such as FRADHPFL, YAEERYPIL, YRGGLEPINF, ESSINF or YQIGL, which were previously produced from OVA by hydrolysis with porcine pepsin for 3 h and also appeared in the hydrolysates produced with simulated fluid (Miguel et al,. 2004; García-Redondo et al., 2010).

Following *in vitro* duodenal hydrolysis with trypsin (E:S 40 U/mg) and chymotrypsin (E:S 0.5 U/mg), the appearance of digestion products of ~20,700, 17,000, 12,500, 10,400 and 6,500 Da has been described (Dupont et al., 2010a), which could coincide with those formed during duodenal digestion with the simulated fluid in our study (Figure 11c). These degradation products were absent from the gastroduodenal digests produced with human duodenal fluid (Figure 11a), as this caused a much more extensive proteolysis of the gastric digests than the simulated fluid. As mentioned in section 4.1, a difference between both duodenal digestion systems laid in the concentration of bile salts, which was much lower in the pool of human fluids, probably due to the prolonged fasting that preceded their extraction. It has been found that bile salts, at the concentration present in the simulated duodenal fluid, which is typical of a fed state, considerably increase the proteolysis of OVA by pancreatic enzymes (Martos et al., 2010). However, despite the lower concentration of bile acids in the human duodenal fluid, it promoted very effectively the proteolysis of intact OVA and of the peptides generated from it by pepsin hydrolysis. This could be attributed to differences in the specificity between human trypsin and α-chymotrypsin and their bovine counterparts, as well as to the presence of peptidases in the human pancreatic extracts (Lyons and Fricker, 2011). In fact, it has been reported that intact OVA and its 40 kDa fragment partially resist hydrolysis by porcine trypsin and chymotrypsin, but they are more readily degraded by an enzyme preparation from the pig pancreas gland that, in addition to both proteases, contains amino and carboxypeptidases (Martos et al., 2010). Furthermore, comparison of the peptide patterns produced by both systems showed that *in vitro* gastroduodenal digestion with human fluids released fewer and shorter peptides, revealing a most

extensive liberation of free amino acids from the short fragments previously formed during the gastric phase (Figure 13 and Annex 8).

In accordance with the least extensive proteolytic degradation, and although there were, as expected, differences in donor's specificity (Mine and Rupa, 2003), inhibition ELISA with individual sera from allergic patients revealed a comparatively higher IgE-reactivity to the gastroduodenal digests produced with simulated fluids (Figure 12). This is probably because of the presence, in addition to intact OVA and its 40 kDa fragment, of a strong IgE-binding band of ~21 kDa and of other minor immunoreactive degradation products (Figure 11d). This pattern is similar to that previously observed after *in vitro* digestion of egg-white with simulated gastroduodenal fluids that allowed the identification of several IgE-binding fragments with molecular masses in the range of 35-20 kDa, as well as others of 15 and 11 kDa, which derived from the hydrolysis of OVA (Martos et al., 2013). The corresponding digests produced with human fluids showed fainter and lower molecular mass IgE-binding degradation products and the virtual absence of intact protein and its high molecular mass fragment (Figure 11b).

In addition, several IgE-binding epitopes were detected among the peptide fragments of molecular mass lower than 3 kDa identified in the digests by RP-HPLC-MS/MS (Figure 13). Considering the binding frequency to human sera of the 27 synthetic peptides that mimicked the products of the gastrointestinal digestion, 4 main areas of the protein bound IgE from more than 40% of the egg allergic patients: 125-176, 188-198, 326-336 and 370-385. Within the first one, 125-176, at least five distinct epitopes were found, including OVA (125-134) and OVA (159-172), as well as OVA (141-154) and OVA (164-176), which were present in the gastrointestinal digests produced with simulated and human fluids, respectively. The peptides OVA (188-198) and OVA (326-

336), found in the gastrointestinal digests produced with simulated fluids, that bound, respectively, 60 and 50% of the sera from allergic patients, are related to previously defined allergenic epitopes. OVA (188-198) partially coincides with one of the five dominant human IgE-binding epitopes described by Mine and Rupa (2003), (38-49, 95-102, 191-200, 243-248 and 251-260), while OVA (326-336) is comprised within the fragment 323-339, considered an immunodominant B and T cell epitope (Elsayed et al., 1991). The discovery of this latter epitope gave rise to a transgenic mouse carrying the MHC class II restricted rearranged T cell receptor transgene, Tg(DO11.10)10Dlo, specific for OVA(323-339), broadly used in *in vitro* studies of T cell differentiation and *in vivo* studies of tolerance and immune responses (Sato et al., 1994).

Finally, the C-terminal fragment, OVA (370-385), showed the highest binding frequency, reacting with 80% of the sera. Human IgE-binding has been demonstrated to broader areas of the C-terminal region of OVA, such as 301-385 (Kahlert et al., 1992) and 347-385 (Honma et al., 1996). On the other hand, the existence of, at least, two epitopes within the shorter fragment OVA (367-385) was implied from a positive basophil stimulation in one allergic patient (Honma et al., 1996). Interestingly, the peptide OVA (375-384) was recognized by IgE from orally sensitized BALB/c mice but not from mice submitted to intraperitoneal or subcutaneous immunization (Mine and Yang, 2007) what suggests that, even if it is protected within the native structure (Figure 14), it can be specifically exposed as a result of digestion. This immunoreactive fragment was present in the gastrointestinal digests produced with simulated fluids, but it could not be found in the gastrointestinal digests produced with human fluids.

Figure 14. Representation of the IgE-binding epitope OVA (370-385) in the 3D structure of the protein. Molecular graphics and analyses were performed with the UCSF Chimera package (Pettersen et al., 2004). The X-ray coordinates (Stein et al., 1991) were obtained at the RCSB Protein Data Bank (PDB ID: 1OVA).

In agreement with the previous studies comparing the *in vitro* digestibility of bovine β-CN and β-LG with human and simulated digestive fluids (Sections 4.1 and 4.2), and despite human fluids led to a much faster proteolytic degradation, a high degree of similarity within the peptide patterns arising from gastric digestion was detected, which was not so evident following duodenal digestion, likely because of the presence of active exopeptidases in the human duodenal extract. The accumulation of large degradation fragments and low molecular mass peptides with IgE-binding properties could contribute to the stronger residual IgE-binding capacity of the gastroduodenal digests produced with simulated fluids as compared with those produced with human fluids, although the gastrointestinal digests produced with human fluids also contained IgE-binding epitopes that could play a role in the allergenic potential of OVA. Among the epitopes released, OVA (370-385) seems to be of special importance, as it was able to bind the IgE in 80%

of the sera employed in the study. More research is needed in order to elucidate the biological activity of this fragment.

OVOMUCOID

4.4. Influence of the carbohydrate moieties on the immunoreactivity and digestibility of the egg allergen ovomucoid

Ovomucoid (OM, Gal d 1) makes up to 11% of the egg white and it is considered the immunodominant allergen as judged by its binding frequency to IgE from allergic patients. OM from hen egg is a glycoprotein with trypsin inhibitor activity, a molecular mass of approximately 28.0 kDa and an isoelectric point of 4.1. Its polypeptide chain consists of 186 amino acids, forming three structurally independent tandem domains each of 60 amino acids in length (Mine and Zhang, 2008). Each domain bears multiple conformational and linear epitopes that are recognized by IgE antibodies from egg allergic patients (Cooke and Sampson, 1997).

One particular characteristic of OM is its high carbohydrate content, which is between 20–25%, with two carbohydrate chains on each of the first and second domains and one chain present on about 50% of the third domain (Mine and Zhang, 2008). Thus, a relevant question that arises is whether the covalently-bound carbohydrate moieties contribute to OM allergenicity. Using sera from allergic patients, Matsuda et al. (1985) reported that, in the third domain, the carbohydrate chain and/or its attachment site could be recognized as antigenic determinants, whereas Zhang and Mine (1998) postulated that the carbohydrate moiety of the third domain rather exerted an inhibiting effect on the IgG and IgE binding properties of OM. Besler et al. (1997) and Cooke and Sampson (1997) concluded that the carbohydrate residues did not take part in the allergenic structures of OM. Hence, the issue of the relevance of the carbohydrate moiety of OM on its potential to sensitize or elicit an allergic response is still under debate.

In the case of many food allergies, particularly to plant proteins, antibodies specific to carbohydrate determinants are frequently found, although they appear not to have clinical relevance (Altmann, 2007). In any case, the fact that glycosylation is a common feature to many food allergens has prompted investigations that showed that

glycans may enhance immunogenicity through the activation of innate Th2 responses (Ruiter and Shreffler, 2012). Furthermore, the carbohydrate chains normally exert a stabilizing effect on protein structure, offering protection towards processing and/or gastroduodenal digestion and thus contributing to the allergenic potential (Breiteneder and Mills, 2005). Regarding OM, there are a few studies dealing with the influence of gastrointestinal digestion on its immunoreactivity (Kovacs-Nolan et al., 2000; Takagi et al., 2005; Jiménez-Saiz et al., 2011a), however, the contribution of the glycan moieties to its digestibility has not been addressed.

The general aim of this chapter is to further investigate the role of the carbohydrate moieties of OM in its allergenic properties and digestibility. To that end, OM was enzymatically deglycosilated and, using blood from egg-allergic patients, the IgE-binding, and basophil activation properties of the glycosylated and deglycosylated forms were compared. Glycosylated and deglycosylated OM were hydrolysed with a physiologically relevant model by mimicking three areas of the gastrointestinal tract: the mouth, stomach and small intestine, the nonglycosylated peptides obtained were identified by RP-HPLC-MS/MS and the IgE-binding properties of the most relevant resulting fragments were evaluated.

4.4.1. RESULTS

❖ *4.4.1.1. Deglycosilation of OM*

To perform OM deglycosylation, the enzyme PNGase F was used. Enzymatic deglycosylation was considered more appropriate than chemical deglycosylation to study the immunoreactivity of OM because, in principle, the polypeptide chain remains unaltered and, therefore, the protein retains the integrity of its epitopes. To assess the effectiveness of deglycosylation SDS-PAGE with Coomassie G-250 and periodic acid–schiff staining was conducted. As shown in Figure 15a, OM appeared as a diffuse group of bands from 30 to 50 kDa, together with a protein band of 14 kDa corresponding to hen egg lysozyme, present due to an incomplete purification of the commercial product (Pellegrini et al., 1992). The electrophoretic pattern of OM was modified after treatment with PNGase F. The bands corresponding to dOM showed lower molecular mass and, consequently, higher electrophoretic mobility due to the removal of the carbohydrate chains. The absence of carbohydrate was confirmed by staining the gel with periodic acid-schiff (Figure 15b), which showed no bands in the lane corresponding to dOM.

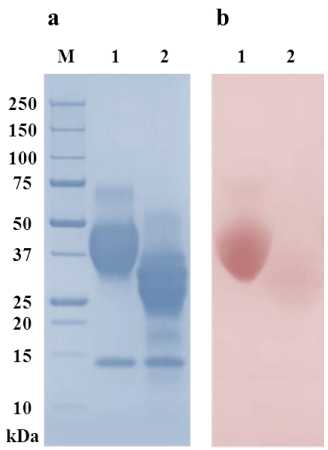

Figure 15. SDS-PAGE patterns, using Bis-Tris 4-12% acrylamide gels, of OM and its deglycosylated form (dOM) with Coomassie blue (a) or PAS (b) staining. M: molecular mass marker; 1: OM, 2: dOM.

We further checked by circular dichroism spectroscopy that, in addition to an efficient removal of N-linked oligosaccharides, PNGase F treatment maintained the degycosylated protein in its native structure. The far-UV circular dichroism spectrum of OM was similar to that reported by Watanabe et al. (1981) and did not change by deglycosylation (Figure 16a). The estimated secondary structure percentages obtained were identical for α-helix (16%) and random coil (36%) features, and slightly different for β-sheet and β-turn (24 and 25% and 23 and 22% for OM and dOM, respectively). The near-UV circular dichroism spectra of native OM was not modified either (Figure 16b), revealing that the secondary and tertiary structures of dOM were not altered when the carbohydrate chains were cleaved.

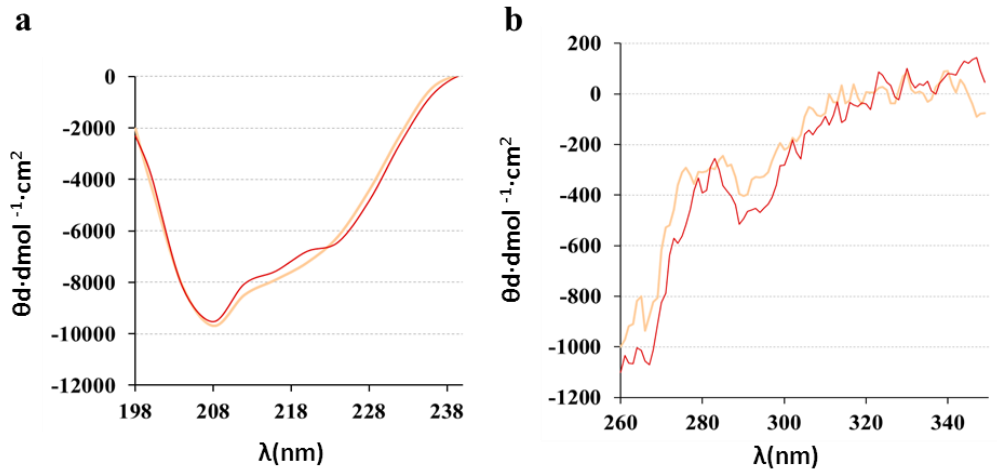

Figure 16. Circular dichroism spectra in the far (a) and near (b) ultra violet region of OM (—) and dOM (—).

❖ *4.4.1.2. In vitro immunoreactivity*

IgE-binding to OM and dOM was evaluated by inhibition ELISA using the sera from 10 egg-allergic patients (nos. 1-10 in Annex 4). Immunoreactivity was expressed as EC50 (effective OM or dOM concentration (µg/mL) for 50% of the maximal IgE binding to OM) and, as depicted in Figure 17, in 8 out of 10 sera the immunoreactivity towards dOM was lower than that of OM.

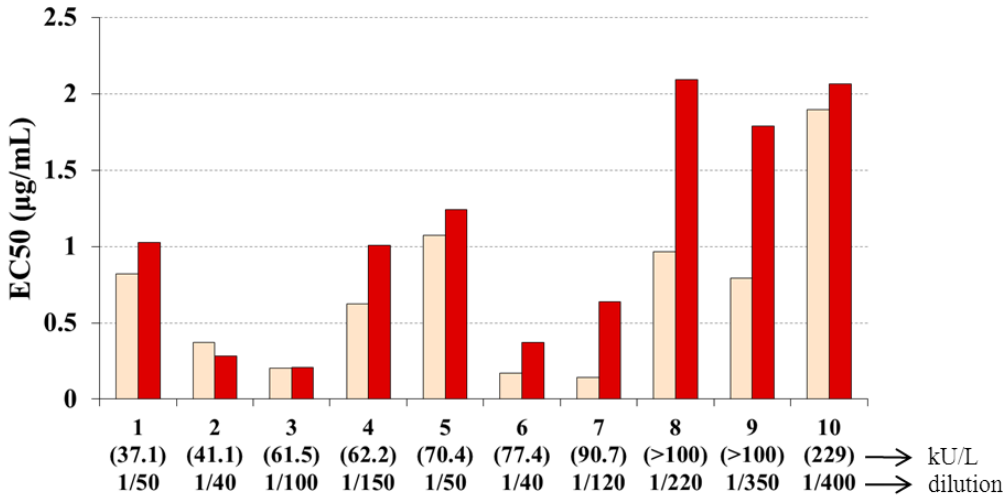

Figure 17. IgE binding of OM (light bars) and dOM (dark bars), expressed as EC50 as estimated by inhibition ELISA with sera from ten allergic patients (nos. 1-10, Annex 4).

In order to investigate the IgE specificity, sera from the same 10 patients were used to perform Western blot analyses (Figure 18b-k). IgE from the 10 sera recognized the bands corresponding to both OM and dOM. However, a previous incubation with dOM completely inhibited the binding to dOM but failed to inhibit the binding to OM in two patients (2 and 6, Figure 18c and 18g) and inhibited the binding to dOM to a much higher extent than that of OM in another two (9 and 10, Figure 18j and 18k). This

provided evidence for the presence of IgE reactive epitopes specific for the glycosylated protein that did not react with the deglycosylated form. In the remaining 6 patients, preincubation with dOM either completely inhibited the IgE reactivity against dOM and OM (patients 1 and 8, Figure 18b and 18i), or partially inhibited both to a similar extent (patients 3, 4, 5 and 7; Figure 18d, 18e, 18f and 18h). The strong reactivity of many of the individual sera towards the contaminating lysozyme was noteworthy.

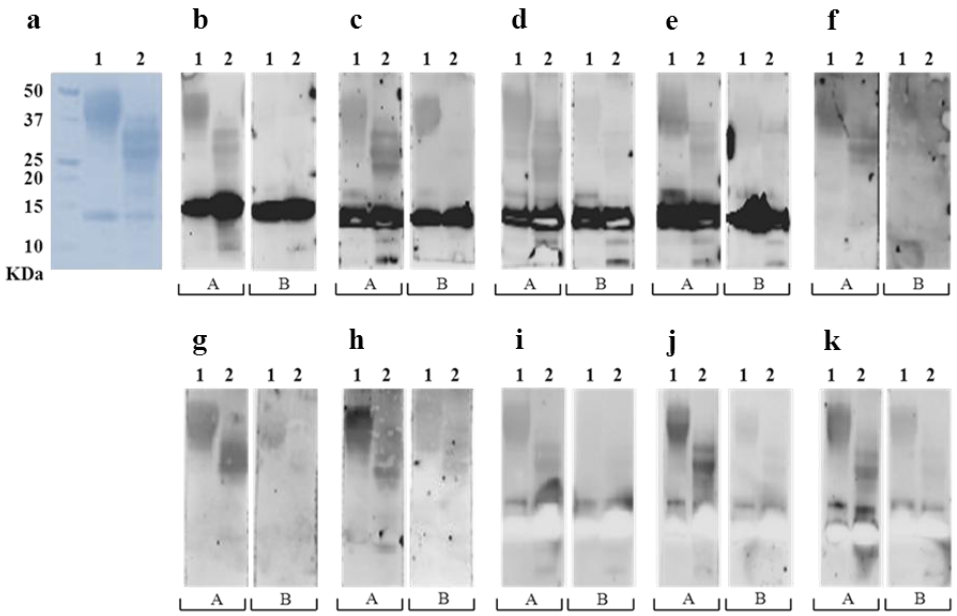

Figure 18. SDS-PAGE (a), using Bis-Tris 4-12% acrylamide gels, and Western blotting (b-k) of OM (1) and dOM (2) with sera from the ten allergic patients (nos. 1-10, Annex 4). Human sera were either not incubated (A) or previously incubated (B) with the amount of dOM calculated to inhibit 100% of IgE binding.

❖ *4.4.1.3. Ex vivo biological activity*

The capacity to trigger basophil activation was determined on PBMCs from 6 egg-tolerant adult donors passively sensitized with a pool of sera from patients 1, 3, 5, 7 and 9

(Annex 4). The results of the basophil activation analysis by flow cytometry are shown in Figure 19. Challenge of basophils with OM and dOM induced activation as measured by upregulation of CD63. Results showed that the removal of the carbohydrate chains moderately reduced the percentage of activated basophils at all the concentrations assayed, although the differences did not reach statistical significance.

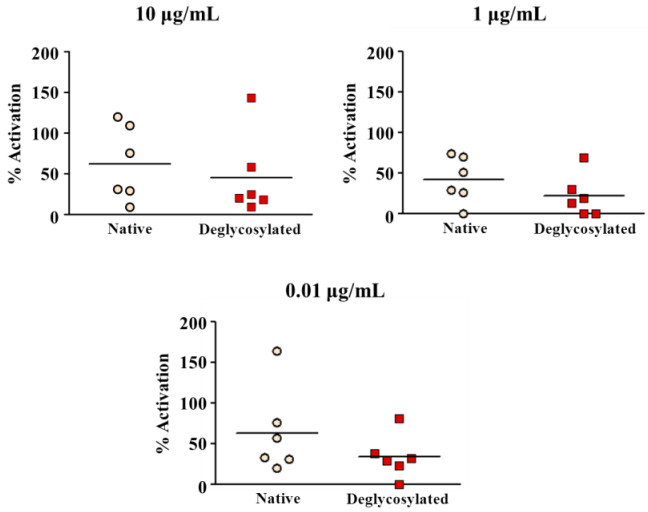

Figure 19. Percentage of activated basophils ($CD63^+CD123^+CD203c^+HLA\text{-}DRlow$) after stimulation with OM and dOM at concentrations between 0.01 to 10 µg/mL. PBMCs from 6 egg-tolerant adult donors were passively sensitized with a pool of sera from 5 children with egg allergy (nos. 1, 3, 5, 7 and 9, Annex 4). Bars indicate the mean of activated basophils.

❖ *4.4.1.4. In vitro simulated orogastrointestinal digestion and immunoreactivity*

Figures 20a and 20b show the SDS-PAGE patterns of OM and dOM following the *in vitro* oral, gastric and duodenal digestions. After the 2 min-oral phase, the SDS-PAGE patterns from both OM and dOM showed a much broader shape, which could be attributed to an incomplete reduction of the nine disulfide bridges caused by the low sample pH (pH 3.5 was used to stop α-amilase action), which diminished their mobility in

the acrylamide gel (Yousif and Kan, 2002). However, as judged by the RP-HPLC pattern (not shown), there were no changes after 2 min of hydrolysis that would point to an unspecific activity of the saliva enzyme.

OM was degraded during the first min of gastric digestion, leaving no intact protein at the end of the gastric phase, and yielding fragments with molecular masses of ~25, ~15 and <10 kDa. All these bands were faintly visible after 60 min of simulated gastric hydrolysis and the lower molecular mass ones (~15 and <10 kDa) persisted throughout the duodenal phase (Figure 20a). dOM was degraded more rapidly and produced bands of ~18, ~ 15 and <10 kDa, with the band corresponding to ~18 kDa being no longer present after the first 7 min of pepsin hydrolysis, so that only the bands corresponding to ~15 and <10 kDa were found at the end of gastric digestion and throughout the subsequent duodenal digestion (Figure 20b).

As shown in Figure 20c and 20d, the ~25, ~15 and <10 kDa fragments formed during gastric digestion of OM and those of ~15 and <10 kDa formed during gastric digestion of dOM were able to bind IgE from a pool of five egg-allergic patients (nos. 1, 3, 5, 7 and 9, Annex 4), although the immunoreactivity of the band of ~15 kDa could be, at least partially, attributed to the presence of residual LYS. Once the duodenal digestion was completed, the bands corresponding to ~15 and <10 kDa, present in OM and dOM digests, still had detectable IgE-binding capacities.

The IgE-binding of the digests was evaluated by inhibition ELISA using the sera from six patients (nos. 11-16, Annex 4). The immunoreactivity decreased to, approximately, 10.6% for OM and 1.2% for dOM at the end of the gastric stage, falling to 4.16% and 1.1% after the duodenal phase for OM and dOM, respectively (not shown).

The lower IgE binding found in the digests of dOM correlated with its highest susceptibility to proteolysis.

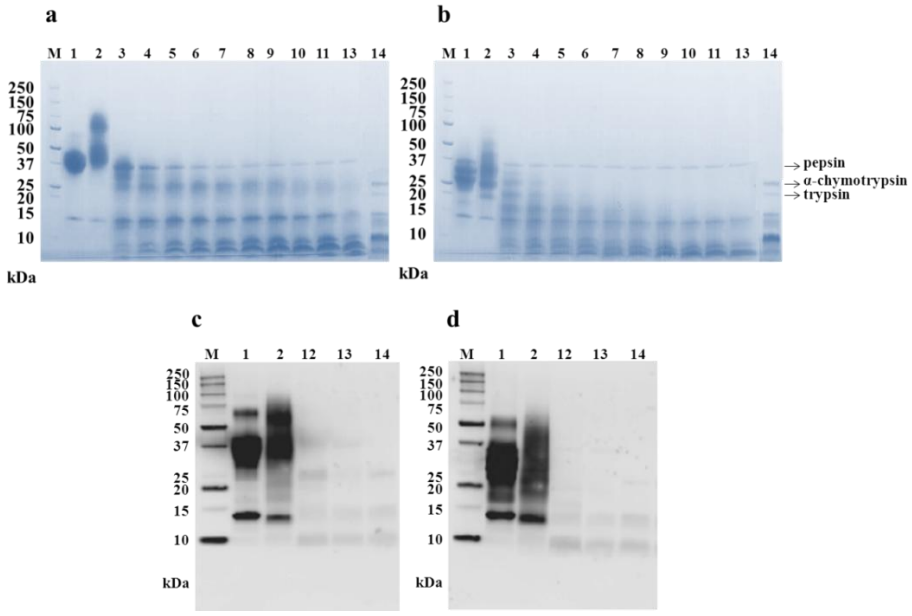

Figure 20. SDS-PAGE patterns (a, b), using Bis-Tris 4-12% acrylamide gels, and Western blotting (c, d) of OM (a, c) and dOM (b, d) after *in vitro* oral, gastric and gastroduodenal digestions. M: molecular mass marker; Lane 1: OM (a, c) and dOM (b, d); lane 2: oral digest; lanes 3-13: gastric digests at 1, 2, 3, 4, 5, 7, 10, 15, 20, 30 and 60-min; lane 14: duodenal digests (60 min of gastric digestion followed by 30 min of duodenal digestion). Membranes were incubated with a pool of sera of five allergic patients (nos. 1, 3, 5, 7 and 9, Annex 4).

❖ *4.4.1.5. Peptide identification after in vitro digestion and epitope recognition*

RP-HPLC-MS/MS was employed to analyze the peptides present in the gastric (60 min) and duodenal (60 min of gastric digestion followed by 30 min of duodenal digestion) digests of OM and dOM and Figure 21 and Annex 9 shows the sequences of

the 146 peptides identified. Only identification of the non-glycosylated fragments was attempted, in view of the difficulty involved in the determination of glycosylated peptides in complex mixtures, because the signal intensity of glycopeptides is low compared to nonglycosylated peptides and often suppressed in their presence (Ongay et al., 2012). Some deglycosylated peptides containing Asn10, Asn53 and Asn75 were detected in the OM hydrolysates (Asn175 is naturally partially glycosylated) probably due to a partial deglycosylation of the commercial sample used. The peptide patterns of the hydrolysates produced were very similar, showing 56 peptides in common after the simulated orogastrointestinal digestion. This indicated that, while deglycosylation enhanced susceptibility to proteolysis, no major differences in the cleavage sites of the protein were detected after hydrolysis times representative of the transit times in the stomach and duodenum. In the N-terminal region, around the glycosylated Asn10, pepsin hydrolysed OM after Ala12 and dOM after Phe8, Asp13 and Glu15. Very few peptides were identified in the region between the glycosilated positions Asn53 and Asn75 even in the deglycosylated form (Figure 21 and Annex 9). In this area, pepsin hydrolysed dOM after His58 and Asp64 and trypsin after Lys63, in both OM and dOM. Met68 was also cleaved in the duodenal digests of dOM. The greatest similarities were found in the third domain, particularly following gastric digestion. Interestingly, peptides with a molecular weight above 2,400 Da only appeared during the digestion of OM, underlining a less extensive proteolytic degradation.

Figure 21. Peptide sequences, identified by RP-HPLC-MS/MS, in the gastric (60 min) and subsequent duodenal digests of OM and dOM (60 min of gastric followed by 30 min of duodenal digestion), and IgE binding ( ), estimated by immunodot with 10 sera of egg-allergic patients (patients 1-10, Annex 4), of 17 synthetic peptides selected within the peptides identified in the simulated digestions. Y: Carbohydrate chains. ⌜⌝ Disulphide bonds.

Considering the similarities between the peptide patterns of the orogastroduodenal digests of OM and dOM, 17 representative peptides, common to both hydrolysates, were selected as explained in the materials and methods section and chemically synthetized. Their IgE binding is shown in Figure 21. The highest IgE-binding (between 70 and 100% of the patients) corresponded to the peptide OM (80-89) and to the region 133-180 [OM (133-148), OM (146-161) and OM (168-180)], moderate binding (40-70% of the patients) to the regions 36-61 [OM (36-45), OM (41-53) and OM (45-61)] and 100-122 [OM (100-110) and OM (109-122)], whereas low binding (<40% patients) was attributed to the peptides OM (25-38), OM (90-103), OM (114-129) and OM (122-140). Peptides OM (19-38), OM (54-68), OM (108-118) and OM (179-186) did not react with any of the 10 patient´s sera employed in the study.

4.4.2. DISCUSSION

OM was successfully deglycosilated using microbial PNGase F (Figure 15a and 15b), an amidase that cleaves between GlcNAc and Asn residues of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins, without secondary or tertiary structural changes as determined by circular dichroism spectroscopy (Figure 16a and 16b). Previous reports, had studied the effect of glycosylation on reduced and alkylated OM (Cooke and Sampson, 1997) or on individual domains of the protein (Matsuda et al., 1985) (Zhang and Mine, 1998). However, because of the importance that conformational epitopes exert in some patients (Cooke and Sampson, 1997; Mine and Zhang, 2002b), the preservation of the structural stability and integrity is very important when assessing the immunoreactivity of OM.

In the present study, 80% of the patients showed lower IgE binding to dOM as compared with OM (Figure 17). The maintenance of the secondary and tertiary structures of the full native form suggests that the differences in IgE reactivity found between the glycosylated and deglycosylated forms are not merely due to changes in protein structure induced by glycans. Furthermore, in the case of some patients, IgE reactivity to OM could not be inhibited by pre-incubation with dOM (Figure 16b-k), what indicates that these patients might be sensitized not only to the peptide epitopes, but also to carbohydrate-containing structures, although cross-reactivity between carbohydrate-containing and amino acid structures could not be excluded. Evidence for the sensitizing potential in humans of glycosylated allergens from major royal jelly proteins, beyond carbohydrate-based cross-reactivity, has been provided (Blank et al., 2012). However, mice subcutaneously sensitized to OM third domains with and without carbohydrate do not show differences in IgE-binding towards each other, showing a high degree of cross-reactivity between the glycosylated and deglycosylated forms (Rupa et al., 2007). At this respect, it should be taken into account that a considerably variability among egg allergic patients in their IgE binding to different linear and conformational epitopes of OM has been described (Cooke and Sampson, 1997; Järvinen at al., 2007), that likely reflects sensitization to different OM forms.

In accordance with the lower IgE binding, we found a subtle reduction in the percentage of activated basophils after incubation with dOM as compared to OM (Figure 19). There are several examples of IgE antibodies against plant food N-glycans that exert biological activity in basophil activation assays (Batanero at al., 1994), but fail to demonstrate their capacity to elicit an immune response and trigger clinical symptoms (Altmann , 2007). On the other hand, other authors have obtained results that support the

immunological and clinical relevance of the carbohydrate determinants in allergens. A recent report from Chiang et al. (2012) states the direct implication of low-molecular-weight oligosaccharides in IgE-mediated anaphylaxis to cow´s milk formula supplemented with prebiotics.

The simulated digestion experiments showed that OM and dOM were not affected by *in vitro* oral digestion, but completely degraded by gastric digestion (Figure 20a and 20b). A profile of 3 main degradation products with molecular masses of ~25, ~15 and <10 kDa was found in the 60 min gastric digests of OM, while dOM was digested more extensively and only bands of ≤ 15 kDa were present at that time. This might be a direct consequence of the removal of the carbohydrate chains that would allow a most efficient hydrolysis by pepsin.

Kovacs-Nolan et al. (2000) described the rapid degradation of intact OM during simulated gastric digestion with the formation of large fragments that could act as allergens, albeit they exhibit reduced IgE-binding activity as compared with the native protein. Two of these, with molecular masses of ~24 and ~18 kDa were identified as OM (21-133) and OM (134-186) (Kovacs-Nolan et al., 2000). The bonds Leu20-Val21 and Ala133-Val134 were cleaved by pepsin in our system (Figure 21) and thus, any of these could correspond with the, likely glycosylated, IgE binding broad ~25 kDa band of the OM digest (Figure 20c) The absence of equivalent bands in the dOM digests after 7 min of hydrolysis could account for their lower immunoreactivity, only attributable to the lower molecular mass products (Figure 20d). Takagi et al. (2005) reported the formation of IgE-binding pepsin degradation products of 7 and 4.5 kDa, what reinforces the hypothesis that patients that positively react to digestion resistant fragments are unlikely to outgrow egg-allergy (Yamada et al., 2000).

Little change in the band pattern was observed during the duodenal phase, likely because the peptides released by pepsin action retain trypsin inhibitory activity that helps to maintain OM peptide fragment integrity (Kovacs-Nolan et al., 2000). Following duodenal digestion, the fragments of ~15 and ≤ 10 kDa that persisted in the digests of OM and dOM could be partially responsible for their residual IgE binding, which was similarly reduced, although not eliminated upon gastrointestinal digestion (Figure 20c and 20d). In any case, IgE-binding of the gastroduodenal digests was very low (<5% of that of the intact protein), what is in agreement with previous results (Jiménez-Saiz et al., 2011a).

A further investigation on whether the peptides resulting from orogastrointestinal digestion of OM contained IgE-binding epitopes revealed that two regions of the protein, that is those within the residues 80-89 and 133-180, were recognized by more than 70% of the allergic patients studied, while from 40 to 70% of the patients recognized two other regions, 36-61 and 100-122 (Figure 21). Most of these high-frequency IgE-binding peptides were either totally or partially coincident with previously described epitopes (Cooke and Sampson, 1997; Zhang and Mine, 1998; Holen et al., 2001; Mine and Zhang, 2002b; Järvinen et al., 2007; Martínez-Botas et al., 2013). However, there are not only numerous IgE binding epitopes distributed along the whole OM structure, but also very many differences in epitope recognition among patients depending on their sensitivity to the allergen (Järvinen et al., 2007). Furthermore, the length of the peptide and the nature of the nearby amino acids also determine the affinity for IgE antibodies. It should be mentioned that longer peptides were found in the gastric and duodenal digests of OM as compared to dOM, due to the more extensive degradation of the latter. In any case, and in view of the peptides identified in the digests of OM and dOM, multiple epitopes within

each domain would remain linked by disulfide bonds despite proteolytic cleavage, giving rise to complex sequences which may have the ability to cross-link several IgE molecules and activate effector cells. Nevertheless, according to Martos et al. (2011), simulated gastrointestinal digestion of OM greatly diminishes its basophyl activating capacity, therefore, considering the allergenicity of OM, the possibility that digestion may promote its sensitizing potential or abrogate its tolerizating capacity should be considered.

In conclusion, this work provides evidence for an enhanced IgE reactivity towards carbohydrate containing OM in some egg-allergic patients that could be due to cross-sensitization, but also to sensitization to the glycosylated components. In addition to a direct implication of the carbohydrate chains of OM on its IgE binding, whose clinical relevance remains to be established, they contribute to an increased resistance to proteolysis, particularly during the first stages of gastric digestion, which may play a role in its allergenic potency. Although the residual IgE binding of the *in vitro* digests of OM and dOM was low, the evaluation of the presence of potential epitopes among the nonglycosylated orogastroduodenal products of digestion of OM and dOM revealed the presence of high-frequency IgE-binding fragments that could remain linked by disulphide bonds.

LYSOZYME

4.5. Digestibility and immunoreactivity of lysozyme

4.5.1. Immunological behavior of *in vitro* digested egg-white lysozyme

Lysozyme (LYS, Gal d 4), is found in many organisms including viruses, insects, amphibians, reptiles, birds and mammals. It appears in a multitude of tissues and fluids, including hen eggs, human milk, tears, saliva and is also secreted by leukocytes (Niyonsaba and Ogawa, 2005). Hen egg is probably the richest source of LYS, containing 1-3 g/L, being egg white LYS one of the best chemically characterized proteins. With 129 amino acid residues (14.3 kDa), an isoelectric point of 10.7 and four disulphide bonds that confer it a stable tertiary structure, LYS has emerged as a model for investigations on protein structure and function.

The structure of LYS, shown in Figure 24, consists of two domains, the α domain comprising residues (1-40 and 90-129) and the β domain (41-89), connected by a long α-helix. The α-domain consists of five α-helical segments shown in purple in Figure 24 (A-E) and the β domain is rich in β sheet structure (Mine et al., 2004) shown in green. The alpha region has two disulphide bridges (Cys 6-Cys 127 and Cys 30-Cys 115), the β domain has another (Cys 64-Cys 80) and the fourth bridge (Cys 76-Cys 94) is located between the two domains (Figure 24). These four disulphide bridges confer high thermal stability to LYS (Jolles and Jolles, 1984). As expected, most of the nonpolar side chains are facing the interior of the molecule, away from contact with the aqueous solvent.

In addition to its valuable biological properties, such as being a potent antibacterial agent against gram-positive bacteria, LYS is also a major allergen in egg white, although its allergenic potential has not been studied in depth and no relevant IgE-binding epitopes have been identified (Mine and Yang, 2008). At least 35% of patients

with clinically observed hen egg hypersensitivity have IgE against LYS (Frémont et al., 1997; Walsh et al., 2005) and this high frequency of sensitization poses a risk, not only when egg is consumed, but also when LYS is used as an antibacterial additive to prevent spoilage of cheese, wine or other food products (Weber et al., 2009).

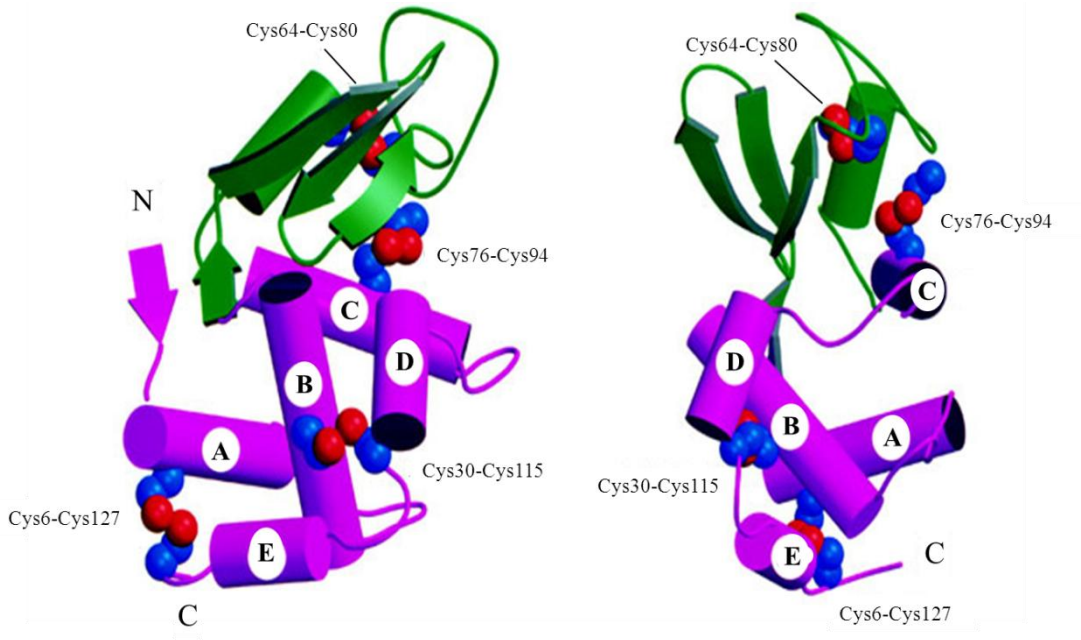

Figure 24. MOLSCRIPT representations of native hen lysozyme showing the α- (purple) and β-domain (green). (Adapted from Van den Berg et al., 1999).

Regarding LYS digestion, previous experiments, aimed at relating the immunogenicity of LYS to its resistance to digestion, showed that *in vitro* gastric proteolysis is extremely pH dependent and only occurs at low pH, yielding peptides with allergenic potential. The remaining intact protein subsequently precipitates at pH values and surfactant concentrations typical of the duodenal medium (Jiménez et al., 2011b).

This would affect the amount of immunoreactive protein that could be absorbed and either sensitize or elicit and allergic reaction.

The section aims to the identification of the peptides produced upon *in vitro* gastrointestinal digestion of LYS, together with their IgE-binding and biological activity, as a contribution to the understanding of what makes LYS a relevant allergen.

4.5.1.1. RESULTS

❖ *4.5.1.1.1. The pH of gastric hydrolysis determines gastroduodenal digestibility and resultant immunoreactivity of LYS*

In vitro gastric digestion was conducted at pH 1.5, 2.0 and 3.2, in the presence and absence of phosphatidylcholine, followed by *in vitro* duodenal digestion, in the presence and absence of bile salts, and the results were monitored by RP-HPLC (Figures 25 and 26) and SDS-PAGE (Figure 27).

LYS was efficiently hydrolysed by pepsin at pH 1.5. There was intact protein left after 30 min of gastric hydrolysis that completely disappeared after 60 min. The presence of phosphatidylcholine slightly protected the protein from the enzyme action, although it did not lead to qualitative changes in the peptide pattern (Figure 25a-d). Gastric digestion released fragments of molecular mass lower than 10 kDa, which were degraded throughout the subsequent *in vitro* duodenal digestion, independently of the absence or presence of phosphatidylcholine (Figure 25e-f, Figure 27a and 27b).

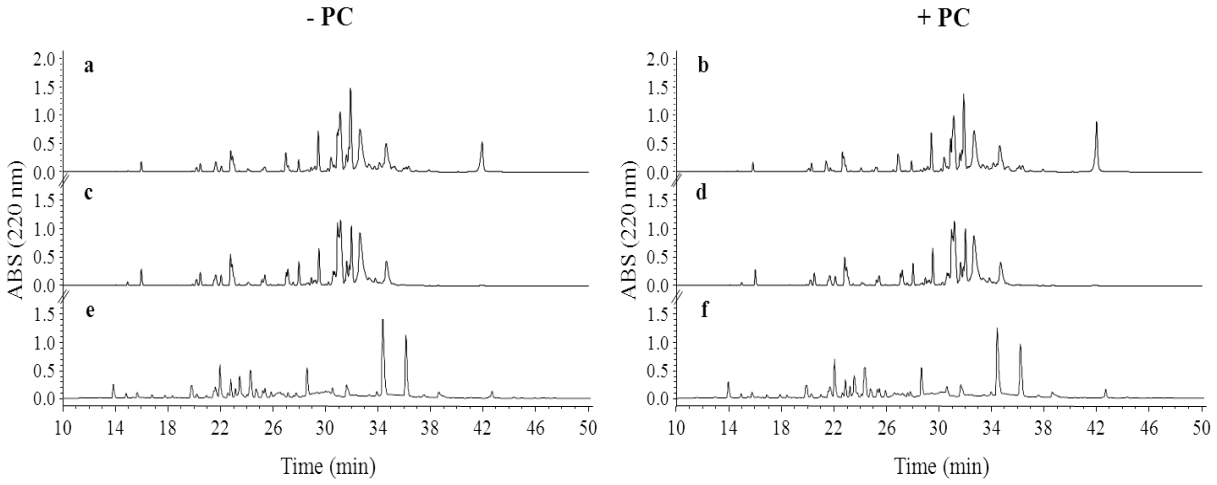

Figure 25. RP-HPLC patterns of LYS subjected to gastric digestion at pH 1.5 for 30 (a, b) and 60 min (c, d), followed by duodenal digestion for 30 min (e, f), in the absence (-PC, left) and presence of phosphatidylcholine (+PC, right).

Pepsin hydrolysed LYS more slowly at pH 2.0 than at pH 1.5 and the protective effect of phosphatidylcholine on LYS degradation was not so apparent, but it gave rise to the same degradation products as determined by RP-HPLC (Figure 26a-d) and SDS-PAGE (Figure 27a). There was intact LYS surviving pepsin action after 60 min at pH 2.0 which precipitated at pH 6.5 in the simulated duodenal medium. After 30 min of *in vitro* duodenal digestion, precipitated LYS was separated by centrifugation and re-solubilized above its isoelectric point. SDS-PAGE analysis of the soluble (Figure 27b) and precipitate fractions (Figure 27c) showed the precipitated protein avoided hydrolysis. However, when phosphatidylcholine was added, it favored LYS solubility and its hydrolysis by pancreatic enzymes, with the appearance of a main degradation product of, approximately, 12 kDa.

When hydrolysis was conducted in a duodenal simulated fluid without bile salts, all LYS remained soluble, being partly hydrolyzed by pancreatic enzymes (Figure 26g). This suggested that the main hydrolysis product of 12 kDa, released during *in vitro* duodenal digestion, was produced from intact LYS by the pancreatic enzymes, an aspect which was further confirmed by hydrolyzing LYS with trypsin and chymotrypsin omitting the previous gastric phase (not shown). In any case, even in the absence of bile salts, when the protein was fully soluble, its proteolysis by trypsin and chymotrypsin was very limited. It is noteworthy that the duodenal peptide patterns subsequent to gastric digestion at pH 2.0 were qualitatively similar to those corresponding to pH 1.5. Furthermore, the enhanced solubility and digestibility of residual LYS in the duodenal medium with added phosphatidylcholine did not lead to the appearance of new or more abundant degradation products (Figure 26 e-f). This indicates that the RP-HPLC peptides detected upon duodenal digestion mainly derived from the peptides previously released from LYS by pepsin action, while partial hydrolysis of the intact LYS surviving gastric digestion, which was restricted to the fraction that remained soluble depending on the medium conditions, mainly yielded the fragment with 12 kDa of molecular mass.

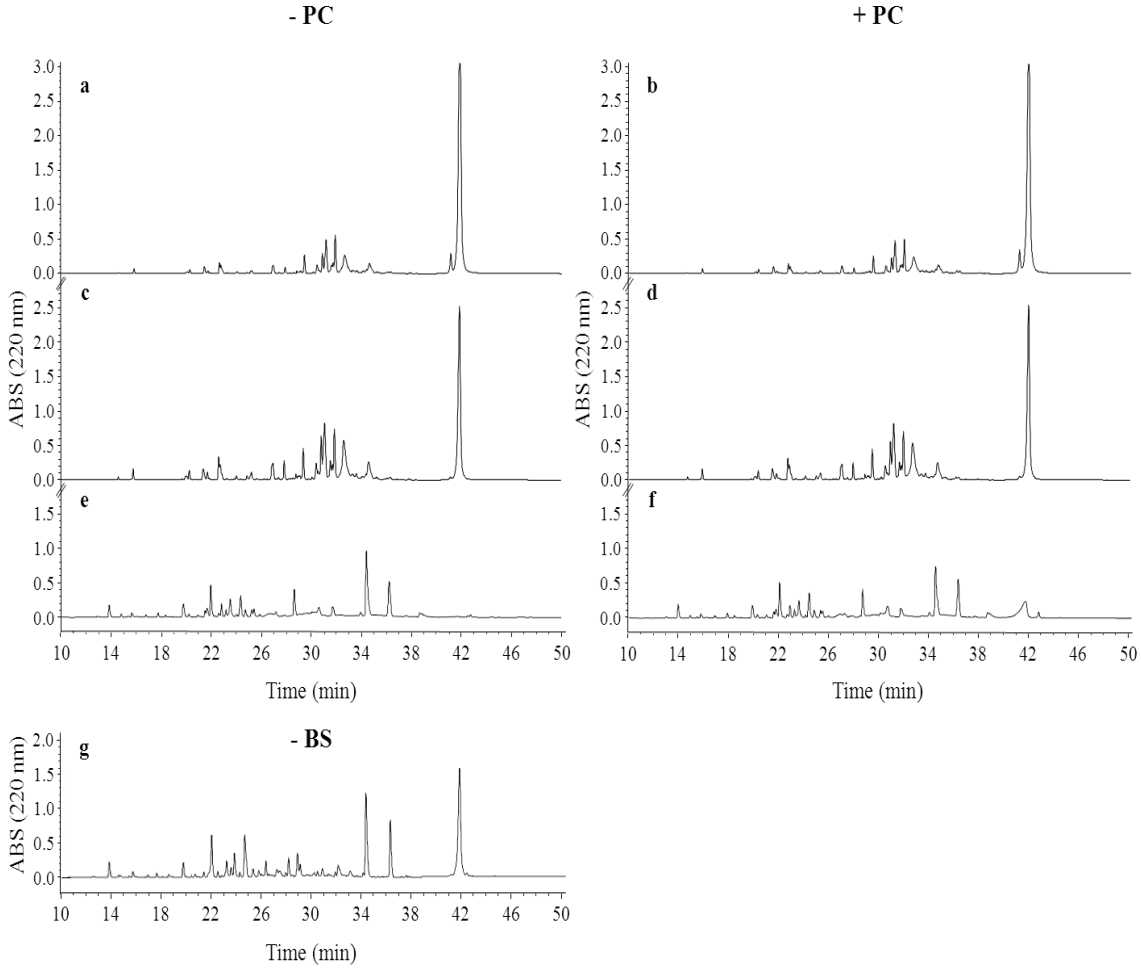

Figure 26. RP-HPLC patterns of LYS subjected to gastric digestion at pH 2.0 for 30 (a, b) and 60 min (c, d) followed by duodenal digestion for 30 min (e, f, g), in the absence (-PC, left) and presence of phosphatidylcholine (+PC, right). Gastroduodenal digests were also conducted in the absence of phosphatidylcholine and bile salts (-BS, g).

Finally, LYS was virtually unaffected by pepsin at pH 3.2 (Figure 27a). The remnant protein precipitated under duodenal conditions, although, in the presence of phosphatidylcholine, soluble intact LYS as well as its 12 kDa fragment became apparent, yet to a lesser extent than when *in vitro* gastric digestion was conducted at pH 2.0 (Figure 27b).

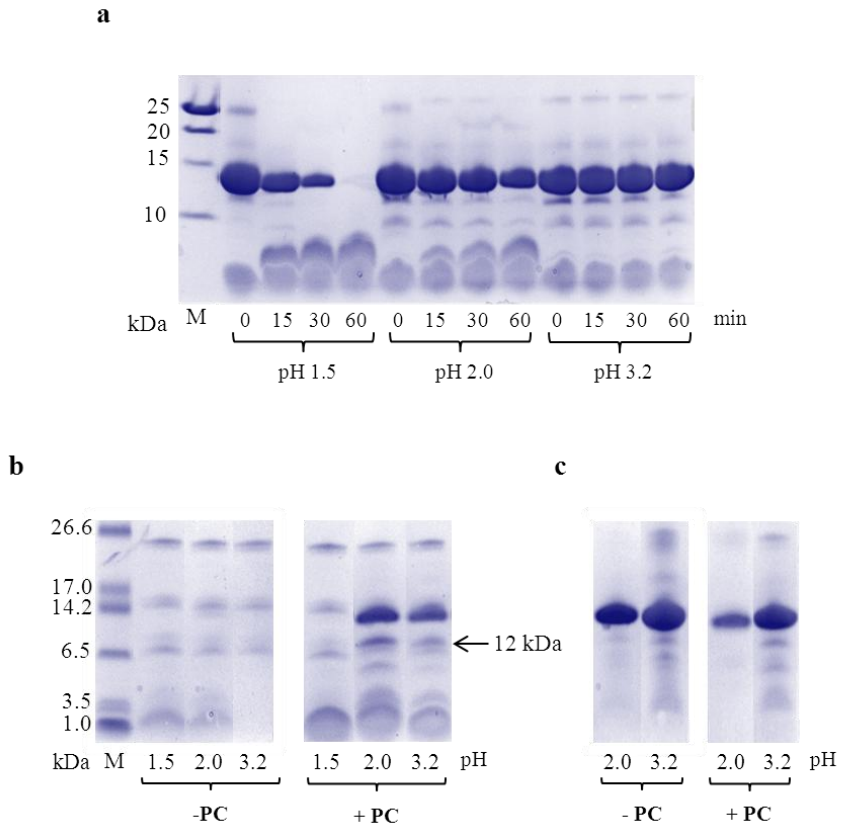

Figure 27. SDS-PAGE patterns under reducing conditions of: (a) LYS subjected to gastric digestion in the presence of phosphatidylcholine at pH 1.5, 2.0 and 3.2, for 0, 15, 30 and 60 min; (b) soluble fraction of LYS subjected to duodenal digestion following gastric digestion at pH 1.5, 2.0 and 3.5 in the absence (-PC) and presence of phosphatidylcholine (+PC); and (c) precipitated fractions (re-solubilized above the isoelectric point of the protein) of the duodenal digests following gastric digestion at pH 2.0 and 3.5, in the absence (-PC) and presence of phosphatidylcholine (+PC). Bis-Tris 12% (a) and Tris-Tricine 16.5% (b, c) acrylamide gels were used. M: molecular mass marker.

Western blotting of the gastric digests was conducted following SDS-PAGE under non-reducing and reducing conditions using a pool of sera from 6 allergic patients (nos. 1-6, Annex 5) (Figure 28). The results indicated that gastric digestion released proteolysis fragments linked by disulfide bridges, showing the appearance of an immunoreactive band of, approximately, 4 kDa after hydrolysis at pH 1.5 and 2.0, albeit only under reducing conditions. Western blotting also showed that intact LYS was more immunoreactive after being reduced (Figure 28b and 28d).

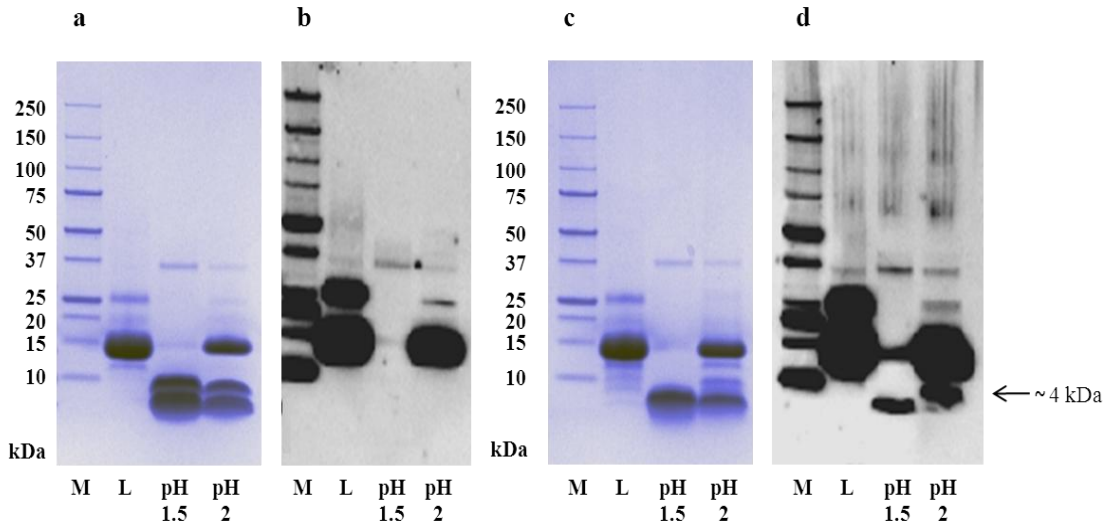

Figure 28. SDS-PAGE patterns, using Bis-Tris 12% acrilamide gels, under non-reducing (a), and reducing (c) conditions, and subsequent Western blotting (b, d) of LYS subjected to gastric digestion in the presence of phosphatidylcholine at pH 1.5 and 2.0 for 60 min. L: intact LYS. M: molecular mass marker. Membranes were incubated with a pool of sera from six allergic patients (nos. 1-6, Annex 5).

As revealed by Western blots of the gastroduodenal digests following SDS-PAGE under reducing conditions, the immunoreactive 4 kDa-band, arising from pepsin hydrolysis at pH 1.5 and 2.0, disappeared upon subsequent duodenal digestion and the new 12 kDa-band, formed from intact LYS, exhibited a considerably IgE-binding (Figure 29). Unlike the sera from non-allergic donors, all the individual sera from egg-allergic patients tested were immunoreactive against LYS and the 12 kDa-band.

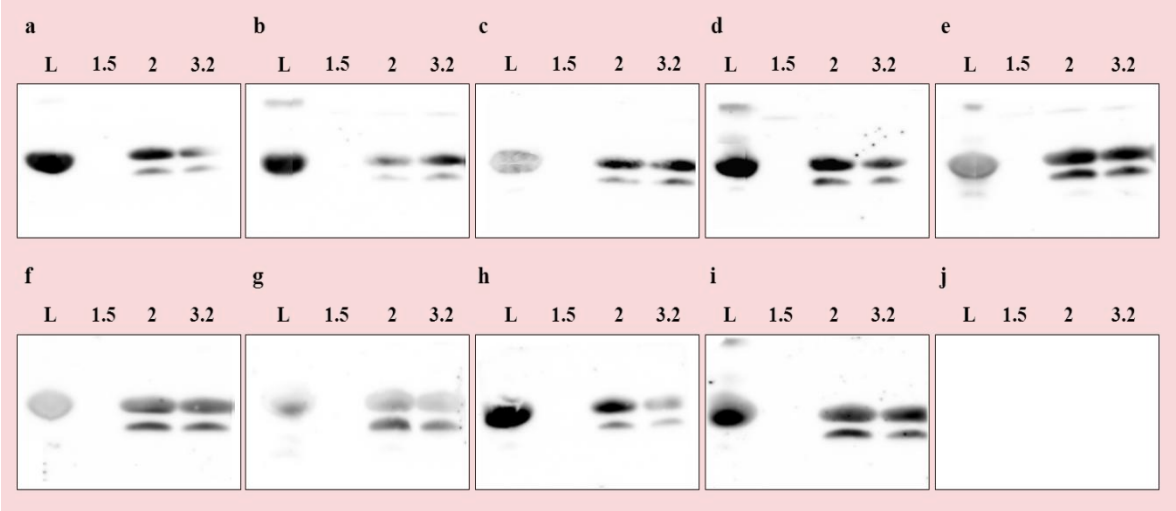

Figure 29. Western blotting subsequent to SDS-PAGE, using Bis-Tris 12% acrilamide gels, under reducing conditions of LYS subjected to duodenal digestion for 30 min, following gastric digestion at pH 1.5, 2.0 and 3.2 for 60 min, in the presence of phosphatidylcholine and bile salts. Individual sera nº 1-9 (Annex 5) (a-i, respectively) and a serum from a non-allergic donor (j) were used. L: intact LYS.

Inhibition ELISA with a pool of sera from allergic patients (nos. 1-6, Annex 5) showed that the gastroduodenal digests following gastric digestion at pH 1.5 and 2.0, and obtained in the absence of both phosphatidylcholine and bile salts, to avoid LYS precipitation that could hinder the quantitative estimation of the resulting

immunoreactivity, still maintained 55 and 35%, respectively, of the IgE binding of the intact protein. This suggested that the lowest gastric pH favored the release of degradation products that could bear IgE-binding epitopes and contribute to the high IgE-binding capacity of the subsequent duodenal digests, despite the absence of intact LYS. In order to estimate the contribution of LYS structure, with 4 disulfide bonds, to its IgE-binding activity, LYS modified by carboxymethylation was also analyzed by inhibition ELISA, showing 20 times more IgE-binding than native LYS (not shown).

❖ *4.5.1.1.2. The gastric and gastroduodenal digestion products of LYS retain IgE-binding epitopes*

Identification of the peptides derived from *in vitro* digestions was attempted by RP-HPLC-MS/MS. The direct analysis of the samples allowed the unambiguous recognition of a limited number of peptides devoid of cysteinyl residues (not shown). Treatment with dithiothreitol gave rise to new peaks, indicating that gastric and subsequent duodenal digestion released many proteolysis fragments linked by disulfide bridges although, due to the complexity in the peptide pattern, it was not possible to trace the appearance of these new fragments to the presence of distinct disulfide bonded sequences before dithiothreitol reduction.

Despite differences in susceptibility to pepsin action depending on the pH, the same peptides were identified in the gastric and gastroduodenal digests following *in vitro* gastric digestion at pH 1.5 and 2.0, though in different amounts. Figure 30 illustrates the sequences arising from gastric digestion at pH 2.0, subsequent duodenal digestion, and

direct duodenal digestion, under conditions avoiding LYS precipitation (without the inclusion of bile salts in the duodenal phase).

The identified peptides produced by pepsin ranged from 803 to 3,077 Da and covered the whole LYS sequence. Taking into account the existence of 4 disulfide bridges in the native LYS structure, connecting Cys6-Cys127, Cys30-Cys115, Cys64-Cys80 and Cys76-Cys94, the possibility arises that larger fragments were formed. In particular, 2 or 3 peptides within the area comprised from residues 53 and 107, which includes Cys64, Cys76, Cys80 and Cys94, could remain linked by 1 or 2 intermolecular disulfide bridges or by 1 intramolecular and 1 intermolecular disulfide bridge, thus forming longer sequences despite the cleavage of several peptide bonds during gastric phase (Figure 30a). Duodenal enzymes released shorter fragments from those previously produced by pepsin that could also be part of bigger disulfide cross-linked peptides as explained above (Figure 30b). In the direct duodenal digestion, and in agreement with their specificities, trypsin hydrolysed LYS after Lys (1, 13, 33, 96, 97) and Arg (21, 45, 61, 73, 114); and chymotrypsin hydrolysed LYS after Tyr (20, 23, 53), Trp (28, 62, 63, 108, 123), Phe (3, 34, 38), Leu (56, 75, 83, 84) and Met (12). There were also peptides corresponding to the cleavage of bonds with Asp (18), Asn (37, 74, 93) and Cys (76, 80) (Figure 30c). Some of these peptides were also found in the gastroduodenal digests.

To identify the LYS fragments that were responsible for IgE binding, as determined by Western blotting, the immunoreactive bands of, approximately, 4 and 12 kDa were subjected to tryptic digestion and MALDI-TOF/TOF. Table 5 shows the tryptic peptides produced by in-gel digestion and Figures 30a-c show the identified LYS fragments. The immunoreactive gastric fragment contained three different products that corresponded to: LYS (11-27), LYS (57-83) and LYS (108-122), which had been

identified by RP-HPLC-MS/MS (Figure 30a) except for the peptide LYS (57-83) that was not within the mass range scanned (100-3,000 m/z). The immunoreactive gastroduodenal fragment, LYS (24-129), of 11.7 kDa, resulted from the chymotrypsin cleavage of Tyr23-Ser24, and probably contained multiple IgE-binding epitopes, as it comprised most of the LYS sequence.

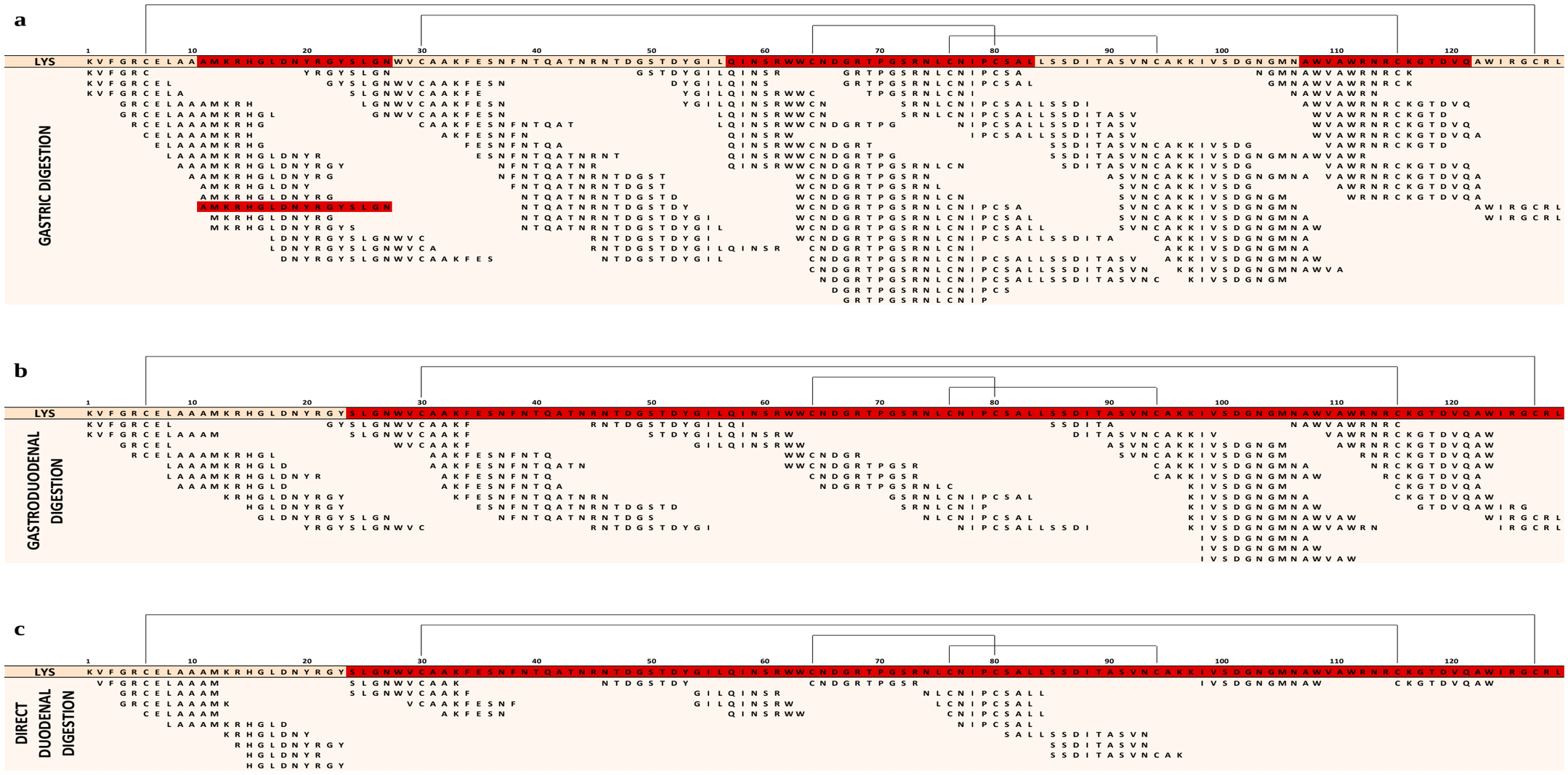

Figure 30. Peptide sequences identified by RP-HPLC-MS/MS in the gastric digests of LYS at pH 2.0 for 60 min (a), subsequent duodenal digest for 30 min (b), and direct duodenal digest at pH 6.5 for 30 min (c), in the absence of phosphatidylcholine and bile salts. The fragments identified by MALDI-TOF/TOF after in-gel tryptic digestion of the LYS bands recognized by human IgE after gastric digestion (Figure 5) and subsequent duodenal digestion (Figure 6) are highlighted in red in the primary sequence of the protein. ⌐¬ : Disulphide bonds.

Table 5. Peptides identified by MALDI-TOF/TOF after in-gel tryptic digestion of the IgE-binding fragments derived from gastric (Figure 5) and subsequent duodenal digestion (Figure 29) of LYS.

	Identified fragment	Tryptic peptide (Ion Mass)	Protein residues	Sequence
Gastric digestion	11-27	1464.712	15-27	HGLDNYRGYSLGN
		1966.995	11-27	AMKRHGLDNYRGYSLGN
	57-83	2089.122	57-73	QINSRWWC*NDGRTPGSR
		1491.820	62-73	WWC*NDGRTPGSR
		1160.558	74-83	NLC*NIPC*SAL
		3231.477	57-83	QINSRWWC*NDGRTPGSRNLC*NIPC*SAL
	108-122	716.335	108-112	WVAWR
		986.600	108-114	WVAWRNR
		1788.908	108-122	WVAWRNRCKGTDVQA
Duodenal digestion	24-129	1105.009	24-33	SLGNWVCAAK
		1428.350	34-45	FESNFNTQATNR
		1752.650	46-61	NTDGSTDYGILQINSR
		992.764	62-68	WWC*NDGR
		515.977	69-73	TPGSR
		2507.463	74-96	NLC*NIPC*SALLSSDITASVNC*AK
		1820.181	97-112	KIVSDGNGMNAWVAWR
		1691.409	98-112	IVSDGNGMNAWVAWR
		1332.535	115-125	C*KGTDVQAWIR
		1044.973	117-125	GTDVQAWIR

C* indicates carbamidomethylated cysteine

❖ *4.5.1.1.3. The gastric and gastroduodenal digestion products of LYS exert biological activity*

LYS and its digests were tested in basophil activation assays and used to stimulate peripheral blood mononuclear cells of egg allergic patients. The results shown in Figure 31 refer to basophils from a non-allergic donor, passively sensitized with a pooled sera from 5 egg allergic patients (nos. 4, 7, 10, 11 and 12, Annex 5), stimulated with intact LYS and with its gastric (pH 1.2 and 2.0 for 60 min) and subsequent duodenal digests (pH 6.5 for 30 min) obtained in the absence of phosphatidylcholine in the gastric phase and bile salts in the duodenal phase. The intact protein and its digests induced basophil activation to practically the same extent, as judged by the enhanced expression of the basophil activation marker CD63, showing that digestion mimicking gastrointestinal conditions that provided the highest degree of LYS hydrolysis (as when the pH of the simulated gastric fluid was 1.5) did not substantially reduce its basophil activating capacity. There was no activation of the non-stripped basophils of the non-allergic donor after incubation with LYS or its digests (not shown).

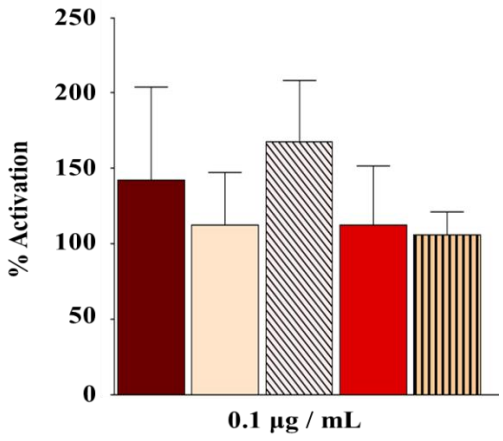

Figure 31. Basophil activating capacity of LYS ( ), its gastric digests obtained at pH 1.5 ( ) and 2.0 ( ) for 60 min and subsequent duodenal digests for 30 min (,), in the absence of phosphatidylcholine and bile salts. The results correspond to basophils from a nonallergic donor, passively sensitized with a pooled serum from five egg-

allergic patients (nos. 4, 7, 10, 11, and 12, Annex 5).

Peripheral blood mononuclear cells from 3 egg allergic (nos. 13, 14 and 15, Annex 5) and non-allergic donors were stimulated with intact LYS and its digests. Proliferation was assessed by the incorporation of MTT. No proliferation was detected in peripheral blood mononuclear cell from non-allergic donors, while peripheral blood mononuclear cells from allergic patients proliferated more to intact than to digested LYS. The T-cell response towards the gastric digests obtained at pH 1.5 was the highest, decreasing during subsequent duodenal digestion, while the gastric digests at pH 2.0 and subsequent duodenal digests, both containing intact LYS, produced similar responses (Figure 32).

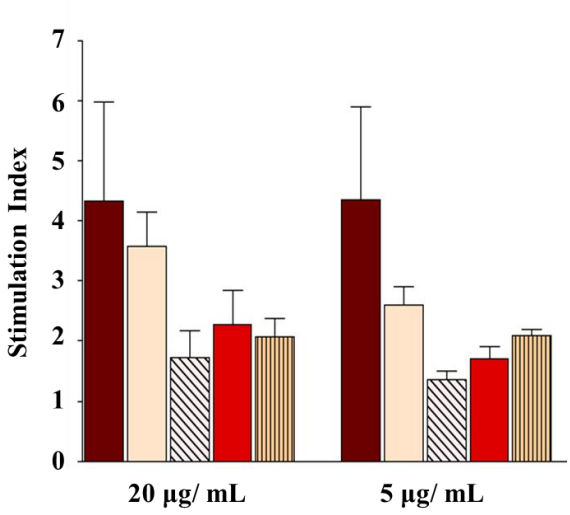

Figure 32. Proliferating responses of peripheral blood mononuclear cells from three allergic patients (nos. 13, 14, and 15, Annex 5) to 5 and 20 µg/mL of LYS ( ), its gastric digests obtained at pH 1. 5 ( ) and 2.0 ( ) for 60 min, and subsequent duodenal digests for 30 min (,) in the absence of phosphatidylcholine and bile salts.

4.5.1.2. DISCUSSION

In agreement with previous results (Jiménez-Saiz et al., 2011b), after 60 min of simulated gastric phase, LYS was completely hydrolysed by pepsin at pH 1.5, partially hydrolysed at pH 2.0 and virtually intact at pH 3.2, and the presence of

phosphatidylcholine, during hydrolysis at pH 1.5 and 2.0, slightly protected the protein from the enzyme action without changing the fragmentation pattern. Intact LYS, surviving gastric digestion at pH 2.0 and 3.2 precipitated under duodenal conditions and phosphatidylcholine partially avoided protein precipitation. We now sought to investigate to what degree the pH of gastric hydrolysis could determine the extent of subsequent duodenal digestion and the immunogenicity and potential allergenicity of LYS. In this study, we found that, despite differences in proteolytic resistance to pepsin depending on the pH of the simulated gastric fluid, which conditioned subsequent proteolysis by pancreatic enzymes, the gastric and gastroduodenal digests maintained allergenic potential, as determined by the basophil activation assay, and preserved T-cell immunogenicity, although to a somewhat lesser extent than the intact protein. These biological activities were specific, because basophils and peripheral blood mononuclear cell from non-allergic donors did not show any reactivity.

Differences in susceptibility to pepsin (Figure 25 and 26), that probably result from a more compact conformation at pH 2.0 than at pH 1.5 that makes LYS a poorer substrate (Polverino de Laureto et al., 2002), gave rise to the same peptide pattern in the gastric and gastroduodenal digests in both cases, as estimated by RP-HPLC-MS/MS, indicating that the increased flexibility at the lowest pH was not accompanied by new cleavage sites. Nevertheless, the pH of gastric digestion greatly conditioned the proportion of LYS that resisted hydrolysis. In this respect, it should be considered that after ingestion of a meal, and because of its buffering effects, the pH of the gastric contents is around 7, decreasing thereafter at a rate that depends on the rate of gastric emptying (which takes on average 48±28 min) and only dropping from around 3 to 1 at the end of this process (Carrière et al., 2001). Therefore, without disregarding the effect

of the food matrix in the extent of LYS digestion, it is likely that a high proportion of intact LYS would reach the duodenum in an *in vivo* situation.

Intact LYS precipitated under duodenal conditions, probably due to electrostatic interaction with negatively charged bile salts at pH 6.5 (Cakir-Kiefer et al., 2011), and this helped it to resist digestion by pancreatic enzymes, as hydrolysis of LYS by trypsin and chymotrypsin occurred only in solution aided by the solubilizing effect of the presence of phosphatidylcholine in the simulated duodenal medium (Figure 27). The nature of the antigen determines its route of uptake, with soluble antigens generally being less immunogenic than denatured or particulate ones, because the latter could use follicle-associated epithelia and M cells to be absorbed, which promotes allergic sensitization (Pabst et al., 2012). However, it is also likely that the insoluble LYS-bile salts complexes are not reabsorbed in the intestine and are eventually excreted in the feces. In addition, even under conditions that favored LYS solubility (such as in the absence or bile salts), it was partially resistant to trypsin and chymotrypsin, leaving intact protein and a high relative mass IgE-binding fragment lacking the N-terminal 1-23 residues. The LYS (24-129) fragment, stabilized by 3 disulfide bridges, that presumably maintains many of the IgE-binding epitopes of LYS, remained unhydrolysed for, at least, 30 min of duodenal digestion, despite possessing several trypsin and chymotrypsin cleavage sites. Given that the reduction of the Cys6-Cys127 S-S bond of LYS results in a very efficient T-cell processing of the C-terminal dominant epitope LYS (107-116) and in a higher intrinsic allergy in BALB/c mice (So et al., 2001), it could be speculated that the release of fragment LYS (24-129), whose C-terminal portion is also free from the body of the protein, may similarly increase LYS allergenic potential.

Structural features of food proteins, such as extensive disulfide bonding, increase protein stability decreasing susceptibility towards proteolytic enzymes. Disulfide bonds are important in the resistance of allergens, such as Ber e 1 from Brazil nut, riboflavin binding protein from egg-white, Act d 2 from kiwifruit and grape lipid transfer protein to digestion (Moreno et al., 2005; Martos et al., 2012; Bublin et al., 2008; Vassilopoulou et al., 2006). In addition, there are several examples of food allergens that, once digested, retain the IgE-binding, basophil mediator release capacity and/or T-cell stimulatory properties of the intact protein, such as Ara h 1 from peanut (Eiwegger et al., 2006), Pers a 1 from avocado (Díaz-Perales et al., 2003) or Act d 1 and Act d 2 from kiwifruit (Bublin et al., 2008), mainly because the proteolytic fragments form stable disulfide-bonded cores. In some cases, it has been described that proteolytic fragmentation can enhance the induction of an effective allergic response, likely because digestion unmasks B-cell epitopes (Pomes, 2010). On the contrary, the easily digestible pollen-related diet allergens, Mal d 1 (apple), Api f 1 (celery) and Cor a (hazelnut) lose their ability to bind IgE and induce basophil degranulation, although they retain activating capacity towards pollen specific T-cells (Schimek et al., 2005).

As shown, LYS, with a high molecular stability due to the presence of 4 disulfide bonds, was very resistant to digestion at pH $\geq$ 2.0 (Figure 27). However, even under conditions that promoted LYS proteolysis, such as when gastric digestion was conducted at pH 1.5, ELISA inhibition assays indicated that the duodenal digestion products maintained a considerable IgE-binding activity and, in fact, these digests cross-linked allergen specific IgE, retaining practically unaltered the basophil activating capacity of the intact protein and thus its allergenic potential (Figure 31). MALDI-TOF/TOF allowed the identification of three possible IgE-binding fragments: LYS (11-27), LYS (57-83) and

LYS (108-122) in an immunoreactive band detected by Western blotting following SDS-PAGE under reducing conditions of the gastric digests obtained at pH 1.5 and 2.0 (Figure 28, 29 and 30 and Table 5. These peptides, which could include relevant IgE-binding epitopes, did not resist subsequent duodenal digestion because they contained several trypsin and chymotrypsin cleavage sites. Considering the peptide profiles arising from RP-HPLC-MS/MS analyses, it is likely that gastric digestion generated nonlinear LYS fragments kept together by disulfide linkages that, in addition to these [particularly LYS (57-83) and LYS (108-122)], could contain similar peptides carrying IgE-binding epitopes, that would not be detected by Western blotting (Díaz-Perales et al., 2003). Furthermore, disulfide linked fragments partially survived subsequent duodenal phase. These peptides could contribute to the strong residual IgE-binding and resulting biological activity of the gastroduodenal digests, compensating for the loss of other epitopes during digestion.

Unfolding by disruption of intramolecular disulfide bonds usually decreases or even abolishes the allergenicity of proteins that display conformational IgE epitopes, which indicates their importance compared to sequential ones (Hazebrouck et al., 2012). The observation that IgE from allergic patients was more reactive to reduced and S-alkylated LYS in inhibition ELISA suggests that reduction of the disulfide bonds increased its IgE-binding capacity, although the possibility that this was due to chemical modification of the epitopes by carboxymethylation cannot be excluded. Similarly, an increased IgE-binding to reduced LYS was detected in Western blots. In contrast, Mine and Zang (2008) reported that partial denaturation of LYS by urea treatment increases its IgE-binding activity, while severe denaturation by reduction and S-alkylation significantly decreases it. Our results suggest that, in addition to the contribution of

conformational epitopes to its allergenic potential, there are relevant IgE-binding epitopes buried within the native structure, that once disclosed by proteolytic degradation could contribute to the allergenicity of the digested protein.

We have found that intact and digested LYS specifically induced proliferation in PBMCs from allergic individuals (Figure 32). According to So et al. (1997), unfolded (reduced and S-alkylated) LYS was 100 times more potent in T-cell stimulation than the native protein, which was associated with a higher susceptibility to be presented by antigen presenting cells. In fact, immunization of BALB/c mice with LYS derivatives with different conformational stability revealed that the least stable derivative led to the most potent Th2 response and IgE production (So et al., 2001). Regarding humans, Holen and Elsayed (1996) detected primary proliferation responses in peripheral blood mononuclear cell from an egg allergic subject on stimulation with LYS but, to the best of our knowledge, there are no reports dealing with the effect of LYS gastroduodenal digestion products on human T-cell proliferation. The observation that the gastric and gastroduodenal digests of LYS induce peripheral blood mononuclear cell proliferation of allergic patients suggests the existence of immunologically active protein fragments that could reach the proximal part of the intestine where most of protein absorption occurs.

In conclusion, *in vitro* gastric and gastroduodenal digests of LYS with different degrees of hydrolysis, depending on the pH of gastric digestion, maintained IgE-binding and basophil activation capacity and preserved T-cell immunogenicity. These biological activities could be attributed to the persistence of intact LYS, due to incomplete gastric degradation and subsequent duodenal precipitation, to the formation of fragment LYS (24-129) by chymotrypsin action on the soluble intact protein or to the release, upon combined gastric and duodenal digestion, of immunoreactive peptides linked by

disulphide bonds. LYS may contain relevant linear epitopes that could be disclosed by digestion increasing its allergenic potential.

4.5.2. Anaphylaxis induced by a drug containing lysozyme and papain. Influence of papain in the IgE response

LYS of egg origin is being increasingly used as an antibacterial additive to prevent spoilage of cheese, wine or other foodstuffs (Iaconelli et al., 2008; Weber et al., 2009), as well as in medicinal products for respiratory tract infections and congestions, which do not usually declare its source (Audicana et al., 2011), thus posing a risk for consumers allergic to hen egg. Hypersensitivity reactions to drugs containing LYS have been described that, in some cases, happen to occur in children that had never intentionally eaten egg and either ignore they are sensitized to egg proteins or react at the first ingestion (Artesani et al., 2008; Ledesma et al., 2007).

This work, conducted in collaboration with the allergy section from the Hospital Infantil Universitario Niño Jesús (Madrid), reports a severe reaction to Lizipaina®, a drug used for the symptomatic relief of mild conditions of the throat and mouth, which contains 5 mg of LYS, in a 15 years old patient able to tolerate, an accumulated dose of at least 60 mg of LYS during a double-blind placebo-controlled food challenge. Furthermore, a successful desensitization after undergoing a rapid oral immunotherapy protocol to hen egg is described.

In addition to LYS, Lizipaina® contains papain (2 mg) and bacitracin (3 mg). Papain is a cysteine proteinase allergen, such as Bromelain and Der p 1, whose intrinsic proteolytic activity has been associated to its capacity to induce Th2-mediated allergic responses (Wills-Karp et al., 2010). This study investigates a possible role for papain in

increasing the allergenicity of LYS through the release of IgE-binding degradation products.

4.5.2.1. RESULTS

❖ *4.5.2.1.1. Case report and oral desensitization*

A 15-year-old boy diagnosed with persistent allergy to egg, fish and tree nuts, atopic dermatitis, rhino conjunctivitis and asthma with sensitization to pollens and epithelia had been previously subjected to a controlled open oral challenge with yolk with a negative result and, since then, the patient had been eating yolk with good tolerance, but avoiding egg in other presentations. In April 2012, he took a tablet of Lizipaina® (5 mg lysozyme, 3 mg bacitracin, and 2 mg papain) due to a mild respiratory viral infection. Fifteen min later, he developed pharyngeal itching, dysphonia, dysphagia, and bronchospasm. These symptoms were controlled with intramuscular epinephrine, intravenous antihistamines and corticosteroids at the Emergency Unit. The boy avoided egg rigorously after the reaction, until attending medical consultation at the allergy section from the Hospital Infantil Universitario Niño Jesús (Madrid) in September 2012.

He was subjected to a double-blind placebo-controlled food challenge with increasing doses of raw dehydrated egg white (OVO-DES NM, Madrid, Spain. 3600 mg corresponding to a whole egg white) after signing an informed consent. The patient developed immediate oral allergy syndrome, and long lasting abdominal pain, pyrosis and diarrhea 1 h after taking a 1,800 mg dose (which corresponded to an accumulated dose of 3,559 mg) with spontaneous clinical resolution.

Table 6. Results of the *in vivo* and *in vitro* tests performed on the allergic patient submitted to a rapid desensitization protocol at different evaluation times before and after oral immunotherapy.

	T_0	T_1	T_2	T_3
Skin prick tests (mm) *				
Egg	5	4	4	6
Egg white	8	3	2	9
Yolk	6	2	2	4
Ovalbumin	5	2	4	4
Ovomucoid	10	3	4	4
Prick-by-prick test with Lizipaina® in saline (mm) *				
	6	8	8	6
Serum total IgE (UI/mL)				
	661	792	609	588
Serum specific IgE (kU/mL)				
Egg	5.95	5.25	6.88	9.95
Egg white	4.72	5.37	6.65	9.32
Yolk	2.18	1.21	1.58	1.54
Ovalbumin	0.76	0.45	0.57	0.68
Ovomucoid	0.92	1.28	1.33	1.44
Lysozyme	4.87	8.59	11.3	14.7
Serum specific IgG4 (mg/mL)				
Egg white	2.37	16.4	23.6	14.6
Ovalbumin	1.79	16.4	25	20.2
Ovomucoid	0.66	4.46	6.49	4.24

Sera from patient before oral immunotherapy (T_0) and after 15 (T_1), 30 (T_2) and 90 days (T_3) of oral immunotherapy.

* Positive if >3.

Then, the patient underwent a rapid desensitization protocol with OVO-DES at the Hospital Allergy Day Unit under the direct supervision of medical and nursing staff. Pre-treatment with 10 mg Cetirizine was conducted the day before initiating the specific oral tolerance induction. A build-up was performed daily, starting with the last tolerated dose in the double-blind, placebo-controlled food challenge. The corresponding doses were administered in sweetened orange juice 1 h apart (900 and 1,800 mg on the 1st day; 1,800 and 3,600 mg on the 2nd day) with no clinical incidences. Cetirizine was discontinued 3 days after reaching the highest dose of raw dehydrated egg white. The patient took 3,600 mg daily during the first week of maintenance at home, and thereafter hen egg was introduced into the patient diet. Given the good clinical evolution, a controlled open food challenge with a tablet of Lizipaina® was carried out after 45 days of maintenance with egg following oral immunotherapy. Increasing amounts were administered every 30 min in 3 doses (0.25, 0.25 and 0.5 of a tablet) to achieve the therapeutic dose. This confirmed tolerance to the drug containing LYS that had elicited the patient's reaction.

The results of the skin prick tests, serum total and specific IgE, and serum specific IgG4 to different egg protein fractions were assessed before (T_0) and after 15 (T_1), 30 (T_2) and 90 days (T_3) of oral immunotherapy (Table 6). In general terms, skin prick tests decreased markedly at T_1, although skin prick tests to egg white increased again at T_3. Prick-by-prick test with Lizipaina® in saline remained positive at the end of the study. Serum IgE specific to whole egg, egg white and, particularly, to LYS continuously increased during the time period considered. Serum IgG4, specific to egg white, OVA and OM also increased, with the highest levels being detected at T_2.

Because the LYS amount that elicited the anaphylactic reaction (5 mg) was much lower than that tolerated during the double blind controlled food challenge, with 900 mg

of dehydrated egg white, corresponding to an accumulated dose of 1,759 mg (approximately equivalent to 30 and 60 mg of LYS, respectively), the possibility that the presence of papain could increase the allergenic potential of LYS was investigated.

❖ *4.5.2.1.2. Reactivity of IgE from egg allergic patients towards lysozyme and Lizipaina®*

Western blotting of LYS, Lizipaina® and egg white was conducted following SDS-PAGE under reducing conditions with sera from the allergic patient at T_0, T_1 and T_2 (Figure 33). Before immunotherapy, the patient's serum was reactive towards the main egg white proteins (Figure 33b, lane 3). LYS, with a molecular mass of 14.3 kDa, was strongly recognized by the serum, both as pure protein (Figure 33b, lane 1) and as part of the egg white (Figure 33b, lane 3). In the Lizipaina® sample there were IgE-reactive bands corresponding to papain (approximately 23 kDa), LYS, and lower molecular mass peptides (Figure 33b, lane 2). Bacitracine, a 1.4 kDa antibiotic peptide, is too small to be resolved and retained in the polyacrylamide gel. The reactivity of the IgE from the patient's sera towards the main egg protein was maintained along immunotherapy, while that towards LYS and the lower molecular mass bands increased, as judged by the progressively stronger staining of the bands (Figure 33 b-d). This is in accordance with the detected increase in the levels of LYS-specific IgE shown in Table 6.

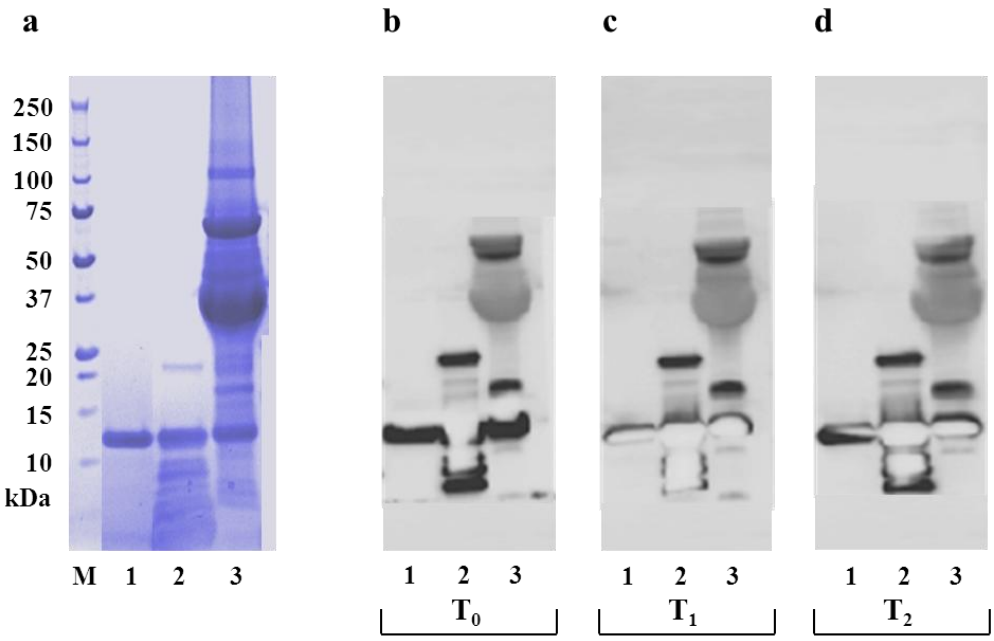

Figure 33. SDS-PAGE patterns (a), using Bis-Tris 12% acrilamide gels, and Western blotting (b, c, d) of LYS (1), Lizipaina® (2) and egg white (3). Electrophoresis was performed under reducing conditions. M: molecular mass marker. Sera from patient before oral immunotherapy (T_0), after 15 days (T_1), and after 1 month (T_2) of oral immunotherapy, were used.

The possibility that the lower molecular mass IgE-binding bands were released from LYS by papain action was tested in an experiment where LYS was incubated with papain for 3 h at 54ºC. SDS-PAGE under reducing conditions showed that papain hydrolysed LYS producing lower molecular mass fragments (Figure 34a, lane 3) which were to a great extent coincident with the bands present in the Lizipaina® sample (Figure 34a, lane 4). According to the Western blot using the serum from patient after 1 month (T_2) of oral immunotherapy, depicted in Figure 2b, these bands strongly bound IgE (Figure 34b, lanes 3 and 4). On the other hand, papain gave, in addition to the 23 kDa band, two other IgE-binding bands of ~15 and ~10 kDa (Figure 34b, lane 1)

.

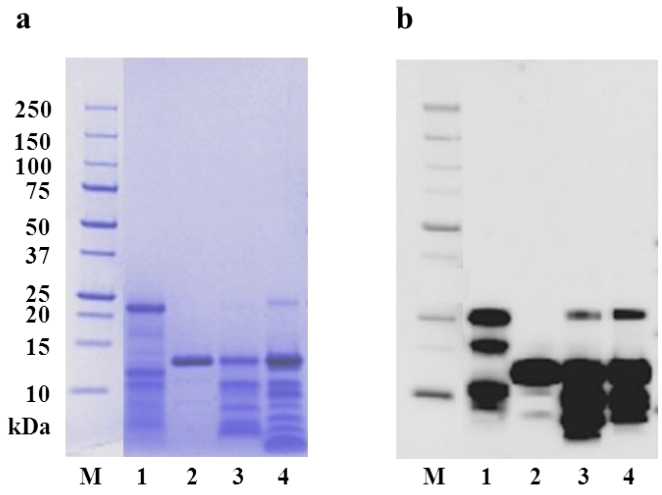

Figure 34. SDS-PAGE patterns (a), using Bis-Tris 12% acrilamide gels, and Western blotting (b) of papain at 0.5 mg/mL (1), LYS at 0.42 mg/mL (2), LYS (0.42 mg/mL) hydrolysed with papain (0.17 mg/mL) at 54ºC for 3h (3) and Lizipaina® (0.5 mg LYS/mL) (4). M: molecular mass marker.

Sera from 5 egg-allergic patients (nos. 10, 16, 7, 17 and 18, Annex 5) were analysed for IgE binding to LYZ, papain and papain treated LYZ, by performing an SDS-PAGE analysis followed by a western blot with and without β-mercaptoethanol (Figure 35). Comparison between the SDS-PAGE patterns under reducing and non-reducing conditions in bis-tris 12% acrylamide gels, showed that the degradation products produced by papain hydrolysis on LYS were only visible after reduction of the disulfide bonds of the protein (Figure 35a). All the allergic donors exhibited IgE reactivity to LYZ, but varied in their reactivity to the bands released by papain action under reducing conditions (Figure 35b-f), which did not seem to depend on the levels of total serum or LYS-specific IgE, except for the patient with the highest titer (266 kU/mL, Annex 5) who exhibited a very strong reaction (Figure 35f). This binding was specific of egg allergy, as no IgE binding was detected when using a serum sample form a non-allergic donor (not shown). All the sera also recognized non-reduced and reduced papain bands, albeit with different intensity.

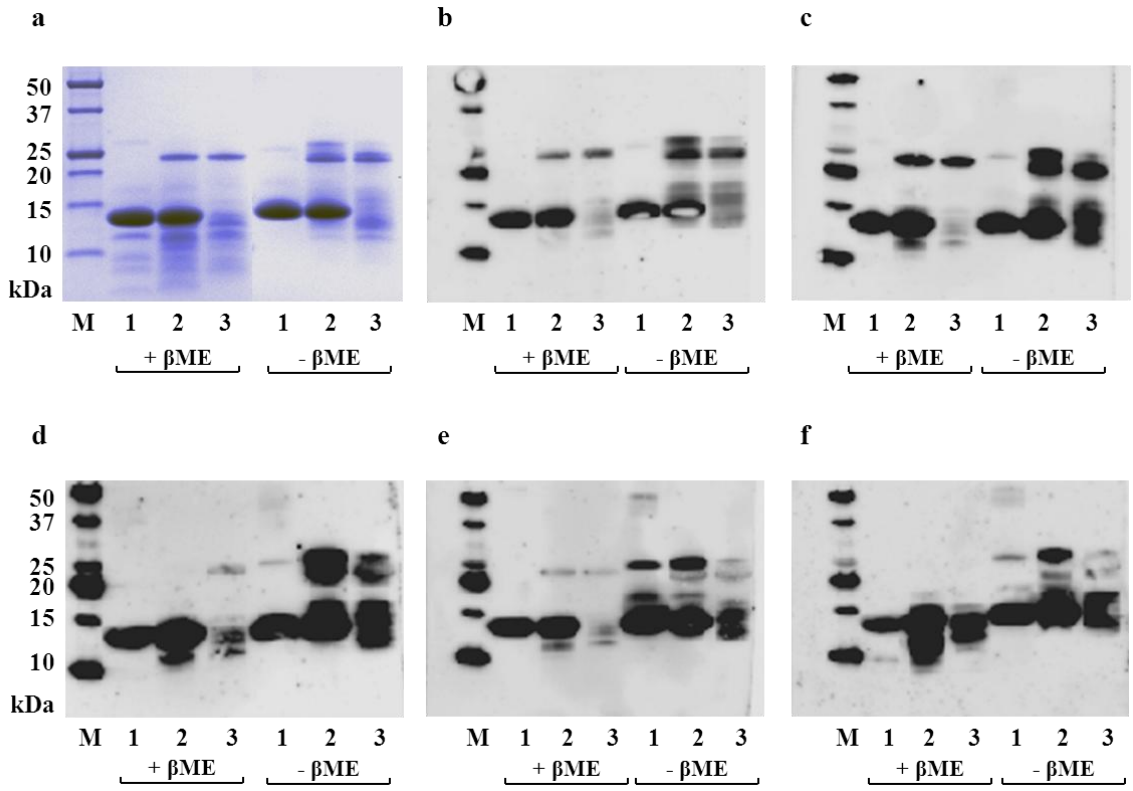

Figure 35. SDS-PAGE pattern (a), using Bis-Tris 12% acrilamide gels, and Western blotting (b-h) with sera from 5 egg allergic patients, (10 (b), 16 (c), 7 (d), 17 (e), 18 (f), Annex 5). Lane 1: LYS; lane 2: LYS hydrolysed with papain at 54ºC for 3h; and lane 3: papain at the same concentration as in lane 2, all dissolved in sample buffer with (+β-ME) and without (-β-ME) reducing agent.

As shown in Figure 36, SDS-PAGE analysis of Lizipaina® was also performed under non-reducing and reducing conditions, using a tris-tricine polyacrylamide gel specific to resolve low molecular mass bands, followed by Western blot (with a pool of 6 sera, nos. 1-6, Annex 5). This confirmed that in the non-reduced sample of Lizipaina®, only a band of intact LYS was present, but when the sample was reduced with β-mercaptoethanol before SDS-PAGE, the band of intact LYS decreased and several degradation products were released (Figure 36), including two IgE-binding fragments of molecular mass > 6,500 Da (Figure 36b,).

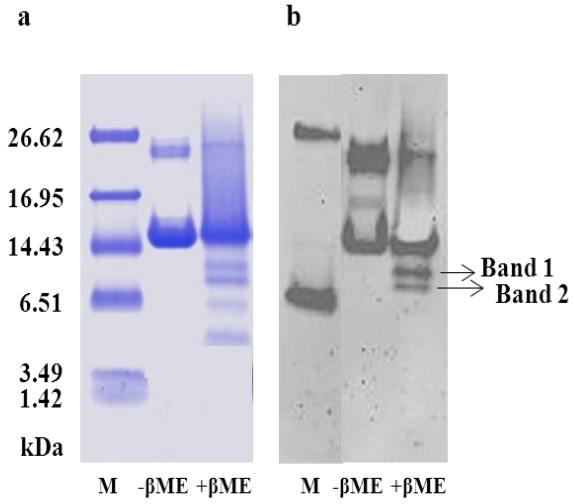

Figure 36. SDS-PAGE patterns (a), using Tris-Tricine 16.5% acrilamide gels, and Western blotting (b) of LYS under nonreducing (-β-ME) and reducing (+β-ME) conditions.

To identify the LYS fragments that contributed to IgE binding, both immunoreactive bands were subjected to in-gel tryptic digestion, as well as intact molecular weight determination by MALDI-TOF/TOF (Table 7).

Table 7. Peptides identified by MALDI-TOF/TOF after in-gel tryptic digestion of the LYS IgE-binding fragments derived from electrophoresis SDS-PAGE under reducing conditions (Figure 4).

	Identified fragment	Tryptic peptide (Ion Mass)	Protein residues	Sequence
BAND 1	22-129	1324.525	22-33	GYSLGNWVC*AAK
		1427.631	34-45	FESNFNTQATNR
		1752.819	46-61	NTDGSTDYGILQINSR
		992.361	62-68	WWC*NDGR
		2506.992	74-96	NLC*NIPC*SALLSSDITASVNC*AK
		1816.992	97-112	KIVSDGNGMNAWVAWR 9: Oxidation (M)
		1691.775	98-112	IVSDGNGMNAWVAWR 8: Oxidation (M)
		1675.783	98-112	IVSDGNGMNAWVAWR
		901.499	106-112	NAWVAWR
		1044.513	117-125	GTDVQAWIR
BAND 2	34-96	1427.734	34-45	FESNFNTQATNR
		1752.961	46-61	NTDGSTDYGILQINSR
		992.445	62-68	WWC*NDGR
		2504.835	74-96	NLC*NIPC*SALLSSDITASVNC*AK
	62-128	992.445	62-68	WWC*NDGR
		2504.835	74-96	NLC*NIPC*SALLSSDITASVNC*AK
		1674.904	98-112	IVSDGNGMNAWVAWR
		1044.61	117-125	GTDVQAWIR

C* indicates carbamidomethylated cysteine

The higher molecular mass band, with an estimated mass of 11,869.889 Da, corresponded to LYS (22-129) (molecular mass 11,887.641 Da). The lower molecular mass band contained two different products (with estimated masses of 7,220 and 7,394 Da) that corresponded, respectively, to LYS (34-96) (6,892) and LYS (62-128) (7,380). Cleavage at Arg_{21}, Lys_{33}, Arg_{68}, Arg_{61}, Lys_{96} and Arg_{128} is consistent with papain specificity, which prefers to cleave at the C-terminal side of Arg or Lys, N-linked to a hydrophobic amino acid residue.

4.5.2.2. DISCUSSION

We report a case of a boy suffering from egg allergy who, once desensitized to egg following a successful rapid oral immunotherapy, could also tolerate a LYS containing drug which had previously caused him a severe allergic reaction. This indicated the effectiveness of the rapid oral desensitization protocol used, although sustained unresponsiveness remains to be established. Successful immunotherapy to egg is associated with increases in specific IgG4, although, as it was the case of the present study (Table 6), this is not always accompanied by decreases in total or specific IgE levels (Burks et al., 2012). Moreover, a slow rate of change in SPT and total IgE, which only decreased significantly after 6 months of tolerance, was reported after a rush protocol (5 days) of desensitization to egg (García-Rodriguez et al., 2011). In our study, even an increase in IgE specific to whole egg, egg white and LYS was detected. Similarly, it was reported that peanut specific IgE increased during the first year of peanut oral immunotherapy, reaching approximately 3-fold after 3 months of treatment, and decreased by 12 to 18 months (Jones et al., 2009).

It was noteworthy that 5 mg of LYS contained in a Lizipaina® tablet caused this patient a severe allergic reaction, while during the double blind placebo controlled food challenge, at least 30 mg of LYS, contained in a single dose of 900 mg of dehydrated egg white (corresponding to an accumulated dose of 1,759 mg containing 60 mg of LYS), were well tolerated. In fact, the IgE present in the sera from this patient reacted more strongly towards Lizipaina® than to an equivalent LYS amount (Figures 33 and 34), and this raised the possibility that the presence of papain could increase the allergenic potential of LYS.

Inhaled papain is not only a potent allergen found in occupational settings (Soto-Mera et al., 2000), but also papain (if enzymatically active, as it is the case of other cysteine proteinases) is able to induce Th2 responses and to exert an adjuvant effect on the sensitizing potential of other allergens, independently of its own antigenicity (Sokol et al., 2008; Cunningham et al., 2012). In addition, its proteolytic properties can enhance its mode and route of transport across the epithelial barriers, which may have an impact on the immune responses generated (Bufe, 1998). Therefore, a role for papain in the initiation of allergic responses to LYS cannot be excluded. In fact, a case of angioedema due to the ingestion of Lizipaina® was reported in a non-egg allergic woman who was regularly eating egg (Pérez-Calderón et al 2007). On the other hand, it is also feasible that papain can increase the eliciting properties of an allergen, such as LYS, in already sensitized patients, by enhancing its bioavailability and the inflammatory response it provokes. Furthermore, the proteolytic action of papain on LYS, described in this work, could boost its allergenicity by producing fragments with increased IgE binding ability. In fact, even if the individual patients tested were shown to vary in their reactivity towards papain-produced LYS fragments, qualitative results suggested that, in some of them, IgE-binding to LYS hydrolysed with papain was more intense than to an equivalent amount of LYS. We had previously found that pepsin digestion of LYS released peptides that carried an important epitope load, so that the IgG and IgE binding properties of the digests were maintained to a high extent (Jiménez-Saiz et al., 2011b; Section 4.5.1).

In fact, the SDS-PAGE patterns of both LYS treated with papain and Lizipaina®, under non-reducing conditions, showed the presence of intact LYS that partially disappeared following reduction with β-mercaptoethanol releasing IgE reactive fragments (Figures 35 and 36). This indicated that papain degraded LYS giving rise to epitopes that

could remain linked through the disulfide bonds present in the LYS molecule. MALDI-TOF/TOF allowed the identification of three possible IgE-binding fragments: LYS (22-129), LYS (34-96) and LYS (62-128) in the immunoreactive bands detected by Western blotting following SDS-PAGE under reducing conditions (Table 7). These peptides comprised the IgE-binding epitopes, described in the previous section (4.5.1), such as LYS (57-83) and LYS (108-122). As illustrated in Figure 37, LYS (22-129) and LYS (62-128) could form part of bigger structures owing to the disulfide bonds connecting Cys_6-Cys_{127}, Cys_{30}-Cys_{115}, Cys_{64}-Cys_{80} and Cys_{76}-Cys_{94}, and the possibility arises that these new structures could present an enhanced allergenic potential.

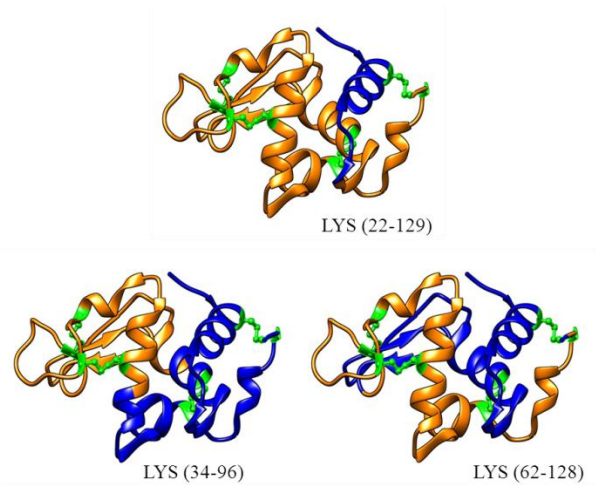

Figure 37. Representation of the identified bands of LYS (Figure 36 and Table 7) in the 3D structure of LYS (orange color). Drawn with UCSF Chimera software (Pettersen et al., 2004) using the crystal structure of LYS described by Naresh et al. (to be published) in the protein data bank website.

A further possibility is that the administration of tablets to be dissolved slowly in the mouth could enhance pregastric absorption of LYS in the oral cavity. These would explain the occurrence of symptoms few min after ingestion of Lizipaina®, as described in this work. Allergen absorption from the human mucosa has been demonstrated (Dirks et al., 2005) and it would be worth investigating if it could be intensified in the presence of proteases. In fact, targeting proteolytic activity by inhibitors has proved potential for adjunct airway allergy therapy (Saw et al., 2012).

In conclusion, the combined administration of lysozyme and papain in drugs may trigger severe allergic reactions that underline the importance of declaring the origin of the active principles. More research is being conducted to determine if specific avoidance of such combinations should be recommended.

5. CONCLUSIONS / CONCLUSIONES

1.- The comparison of two models of digestion using human or simulated fluids on β-LG, β-CN and OVA leaded to similar results respect to hydrolysis patterns and immnunoreactivity of the digests, suggesting that i*n vitro* digestion model with simulated fluids used in this thesis provide a good estimation of the potential allergenicity of protein digests.

2.- Digestion with human and simulated fluids has allowed the identification of 195, 152 and 213 peptides from degradation of β-LG, β-CN and OVA respectively. Several of them are comprised in prominent immunoreactive areas of the proteins detected by peptide microarray and dot-blot corresponding to sequences β-LG (43-62), β-LG (86-100), β-LG (122-147), β-CN (57-68), β-CN (82-93) and OVA (370-385).

3.- The carbohydrate moieties of OM exert a protective effect against proteolysis, particularly during the first stages of gastric digestion, contributing to a higher allergenic potency. Some egg-allergic patients present an enhanced IgE reactivity towards glycated OM compared with deglycosylated OM but seems to have no clinical relevance.

4.- The evaluation of the presence of potential epitopes among the digests of OM and dOM revealed the presence of high-frequency IgE-binding fragments that could remain linked by disulphide bonds. However, the total residual IgE binding of the *in vitro* digests was low.

5.- *In vitro* gastric and gastroduodenal digests of LYS maintain IgE binding and basophil activation capacity and preserve T-cell immunogenicity.

6.- *In vitro* digestion of LYS has allowed the identification of three products with IgE binding capacity, that corresponded to the fragments LYS (11-27), LYS (57-83) and LYS (108-122) in the gastric stage and the fragment LYS (24-129), resulted from the chymotrypsin cleavage of Tyr_{23}-Ser_{24} during duodenal phase. These fragments could be linked by disulphide bonds.

7.- The combined administration of LYS and papain in drugs may trigger and/or enhance severe allergic reactions that underline the importance of understand in depth the interactions between the constituents of drugs or food.

1 -. La comparación de dos modelos de digestión utilizando fluidos humanos en un caso y fluidos simulados en otro, aplicados a importantes alérgenos de huevo y leche como β-Lg, β-CN y OVA ha demostrado que ambos modelos proporcionan una buena estimación de la alergenicidad potencial de la proteína digerida.

2 -. La digestión sobre dichas proteínas, β-Lg, β-CN y OVA, ha permitido la identificación de 195, 152 y 213 péptidos respectivamente, entre los productos resultantes. Se han descrito epítopos con alta capacidad inmunorreactiva, destacando los fragmentos β -LG (43-62), β-LG (86-100), β-LG (122-147), β-CN (57-68), β-CN (82-93) y OVA (370-385).

3 -. Las cadenas de carbohidratos presentes en la estructura del OM ejercen un efecto protector frente a la proteolisis, en particular durante las primeras etapas de la digestión gástrica, lo que puede contribuir a que algunos pacientes alérgicos al huevo presentan mayor reactividad hacia IgE en OM glicosilado en comparación con la forma desglicosilada (dOM).

4 -. La evaluación de potenciales epítopos entre los digeridos de OM y dOM reveló la presencia de fragmentos con alta capacidad de unión a IgE que podrían permanecer unidos por enlaces disulfuro. Sin embargo, la capacidad total de unión a IgE de los digeridos fue baja.

5 -. Los digeridos gástricos y gastroduodenales de LYS mantienen la capacidad de unión a IgE y activación de basófilos e incrementan la proliferación celular.

6.- En la digestión *in vitro* de LYS se han identificado 3 productos con capacidad de unión a IgE, que corresponden a los fragmentos LYS (11-27), LYS (57-83) and LYS (108-122) generados durante la digestión gástrica y el fragmento LYS (24-129), que se obtiene por acción de la quimotripsina sobre el enlace Tyr_{23}-Ser_{24} durante la fase duodenal. Estos fragmentos podrían permanecer unidos por enlaces disulfuro.

7.- La administración conjunta de LYS y papaína en fármacos puede provocar y/o potenciar severas reacciones alérgicas, lo que subraya la importancia de conocer en profundidad las interacciones entre los constituyentes de los medicamentos o de los alimentos.

6. REFERENCES

Adams, S. L., Barnett, D., Walsh, B J., Pearce, R. J., Hill, D. J., Howden, M. E. (1991). Human IgE-binding synthetic peptides of bovine beta-lactoglobulin and alpha-lactalbumin. *In vitro* cross-reactivity of the allergens. *Immunology & Cell Biology*. 69, 191-197.

Albrecht, M., Kuhne, Y., Ballmer-Weber, B. K., Becker, W. M., Holzhauser, T., Lauer, I., Reuter, A., Randow, S., Falk, S., Wangorsch, A., Lidholm, J., Reese, G., Vieths, S. (2009). Relevance of IgE binding to short peptides for the allergenic activity of food allergens. *Journal of Allergy and Clinical Immunology*. 124, 328-336.

Alessandri, C., Sforza, S., Palazzo, P., Lambertini, F., Paolella, S., Zennaro, D., Rafaiani, C., Ferrara, R., Bernardi, M. L., Santoro, M., Zuzzi, S., Giangrieco, I., Dossena, A., Mari, A. Tolerability of a fully maturated cheese in cow's milk allergic children: biochemical, immunochemical, and clinical aspects. (2012). *PLoS ONE*. 7, e40945.

Almaas, H., Cases, A. C., Devold, T. G., Holm, H., Langsrud, T., Aabakken, L., Aadnoey, T., Vegarud, G. E. (2006a). *In vitro* digestion of bovine and caprine milk by human gastric and duodenal enzymes. *International Dairy Journal*. 16, 961-68.

Almaas, H., Eriksen, E., Sekse, C., Comi, I., Flengsrud, R., Holm, H., Jemsem, E., Jacobsen, M., Langsrud, T., Vegarud, G. E. (2011). Antibacterial peptides derived from caprine whey proteins, by digestion with human gastrointestinal juice. *British Journal of Nutrition*. 106, 896-905.

Almaas, H., Holm, H., Langsrud, T., Flengsrud, R., Vegarud, G. E. (2006b). *In vitro* studies of the digestion of caprine whey proteins by human gastric and duodenal juice and the effects on selected microorganisms. *British Journal of Nutrition*. 96, 562-69.

Altmann, F. (2007). The role of protein glycosylation in allergy. *International Archives of Allergy and Immunology*. 142, 99-115.

Anson, M. L. (1938). The estimation of pepsin, trypsin, papain, and cathepsin with haemoglobin. *Journal of General Physiology*. 22, 79-89.

Artesani, M. C., Donnanno, S., Cavagni, G., Calzone, L., D´Urbano, L. (2008). Egg sensitization caused by immediate hypersensitivity reaction to drug-containing lysozyme. *Annals of Allergy, Asthma & Immunology*. 101, 105-105.

Audicana, M. T., Barasona, M. J., Corominas, M., De Barrio, M., García, M. C., García, J. C., Gastaminza, G., Laguna, J. J., Lobera, T., López, M., Martín, J., Moreno, E., Ortega, N., Torres, M. J. (2011). Potential hypersensitivity due to the food or food additive content of medicinal products in Spain. *Journal of Investigational Allergology and Clinical Immunology*. 2, 496-506.

Ball, G., Shelton, M. J., Walsh, B. J., Hill, D. J., Hosking, C. S., Howden, M. E. A. (1994). Major continuous allergenic epitope of bovine beta-lactoglobulin recognized by human IgE binding. *Clinical & Experimental Allergy*. 24, 758-764.

Bannon, G. A. (2004). What makes a food protein an allergen? *Current Allergy and Asthma Reports*. 4, 43-46.

Batanero, E., Villalba, M., Rodríguez, R. (1994). Glycosylation site of the major allergen from olive tree pollen. Allergenic implications of the carbohydrate moiety. *Molecular Immunology*. 1, 31-37.

Baur, X., König, G., Bencze, K., Fruhmann, G. (1982). Clinical symptoms and results of skin test, RAST and bronchial provocation test in thirty-three papain workers: evidence for strong immunogenic potency and clinically relevant "proteolytic effects of airborne papain". *Clinical & Experimental Allergy*. 1, 9-17.

Bergmeyer, H. U., Gawehn, K., Grassl, M. (1974). In Methods of Enzymatic Analysis. Volume I, 515-516. Academic Press, Inc. New York, NY.

Berin, M. C., Sampson, H. A. (2013). Food allergy: an enigmatic epidemic. *Trends in Immunology*. 34, 390-397.

Bernard, T. S. (1953). Human salivary amylase secretion in relation to diet. *The Journal of Physiology*. 119, 153-156.

Besler, M., Steinhart, H., Paschke, A. (1997). Allergenicity of hen's egg-white proteins: IgE binding of native and deglycosylated ovomucoid. *Food and Agricultural Immunology*. 9, 277-288.

Besler, M., Steinhut, H., Paschke, A. (2001). Stability of food allergens and allergenicity of processed foods. *Journal of Chromatography B*. 756, 207-228.

Blank, S., Bantleon, F. I., McIntyre, M., Ollert, M., Spillner, E. (2012). The major royal jelly proteins 8 and 9 (Api m 11) are glycosylated components of Apis mellifera venom with allergenic potential beyond carbohydrate-based reactivity. *Clinical & Experimental Allergy*. 42, 976-985.

Bøgh, K. L., Barkholt, V., Rigby, N. M., Mills, E. N. C., Madsen, C. B. (2012). Digested Ara h 1 loses sensitizing capacity when separated into fractions. *Clinical and Experimental Allergy*. 60, 2934-2942.

Boisen, S., Eggum, B. O. Critical evaluation of in vitro methods for estimating digestibility in simple-stomach animals. (1991). *Nutrition Research Reviews*. 4, 141-162.

Bossios, A., Theodoropoulou, M., Mondoulet, L., Rigby, N. M., Papadopoulos, N. G., Bernard, H., Adel-Patient, K., Wal, J. M., Mills, C. E. N., Papageorgiou, P. (2011). Effect of simulated gastro-duodenal digestion on the allergenic reactivity of beta-lactoglobulin. *Clinical and Translational Allergy*. 1, 6.

Bouzerzour, K., Morgan, F., Cuinet, I., Bonhomme, C., Jardin, J., Le Huerou-Luron, I., Dupont, D. (2012). *In vivo* digestion of infant formula in piglets: protein digestion kinetics and release of bioactive peptides. *British Journal of Nutrition*. 108, 2105-2114.

Boyce, J. A., Assa'ad, A., Burks, A. W., Jones, S. M., Sampson, H. A., Wood, R. A., Plaut, M., Cooper. S. F., Fenton, M. J., Arshad, S. H., Bahna, S. L., Beck, L. A., Byrd-Bredbenner, C., Camargo, C. A. Eichenfield, L., Furuta, G. T., Hanifin, J. M., Jones, C., Kraft, M., Levy, B. D., Lieberman, P., Luccioli, S., McCall, K. M., Schneider, L. C., Simon, R. A., Simons, F. E., Teach, S. J., Yawn, B. P., Schwaninger, J. M. (2010). Guidelines for the Diagnosis and Management of Food Allergy in the United States: Summary of the NIAID-Sponsored Expert Panel Report. *Journal of Allergy and Clinical Immunology*. 126, 1105-1118.

Branum, A. M., Lukacs, S. L. (2009). Food allergy among children in the United States. *Pediatrics*. 124:1549-55.

Breiteneder, H., Mills, E. N. C. (2005). Molecular properties of food allergens. *Journal of Allergy and Clinical Immunology*. 115, 14-23.

Bublin, M., Radauer, C., Knulst, A., Wagner, S. Scheiner, O., Mackie, A. R., Mills, E. N. C., Heimo Breiteneder. (2008), Effects of gastrointestinal digestion and heating on the allergenicity of the kiwi allergens Act d 1, actinidin, and Act d 2, a thaumatin-like protein. *Molecular Nutrition & Food Research*. 52, 1130-1139.

Bufe, A. (1998). The biological function of allergens: relevant for the induction of allergic diseases? *International Archives of Allergy and Immunology*. 117, 215-219.

Burks, A. W., Jones, S. M., Wood, R. A., Fleischer, D. M., Sicherer, S. H., Lindblad, R. W., Stablein, D., Henning, A. K., Vickery, B. P., Liu, A. H., Scurlock, A. M., Shreffler, W. G., Plaut, M., Sampson, H. A. (2012). Oral Immunotherapy for Treatment of Egg Allergy in Children. *The New England Journal of Medicine*. 367, 233-243.

Cakir-Kiefer, C., Miclo, L., Balandras, F., Dary, A. Soligot, C., Le Roux, Y. (2011). Transport across caco-2 cell monolayer and sensitivity to hydrolysis of two anxiolytic peptides from α_{S1}-casein, α-casozepine, and α_{S1}-casein-(f91-97):

effect of bile salts. *Journal of Agricultural and Food Chemistry*. 59, 11956-11965.

Carrière, F., Renou, C., Ville, E., Grandval, P., Laugier, R. (2001). Advantage of expressing the variations in some digestive parameters as a function of gastric emptying instead of time. *Digestion.* 64, 46-53.

Cerecedo, I., Zamora, J., Shreffler, W. G., Lin, J., Bardina, L., Dieguez, M. C., Wang, J., Muriel, A., De la Hoz, B., Sampson, H. A. (2008). Mapping of the IgE and IgG4 sequential epitopes of milk allergens with a peptide microarray-based immunoassay. *Journal of Allergy and Clinical Immunology*. 122, 589-594.

Chatchatee, P., Järvinen, K. M., Bardina, L., Vila, L., Beyer, K., Sampson, H. A. (2001). Identification of IgE and IgG binding epitopes on beta- and kappa-casein in cow's milk allergic patients. *Clinical and Experimental Allergy*. 31, 1256-1262.

Chatterton, D. E. W., Rasmussen, J. T., Heegaard, C. W., Sorensen. E. S., Petersen, T. E. (2004). *In vitro* digestion of novel milk protein ingredients for use in infant formulas: Research on biological functions. *Trends in Food Science & Technology*. 15, 373-383.

Chiang, W. C., Huang, C. H., Llanora, G. V., Gerez, I., Goh, S. H., Shek, L. P., Nauta, A. J., Van Doorn, W. A., Bindels, J., Ulfman, L. H., Knipping, K., Delsing, D. J., Knol, E. F., Lee, B. W. (2012). Anapylaxis to cow's milk formula containing short-chain galacto-oligosaccharide. *Journal of Allergy and Clinical Immunology*. 130, 1361-1367.

Chicon, R., Belloque, J., Alonso, E., López-Fandino,R. (2008). Immuno reactivity and digestibility of high-pressure-treated whey proteins. *International Dairy Journal*. 18, 367-376.

Chirino, A. J., Ary, M. L., Marshall, S. A. (2004). Minimizing the immunogenicity of protein therapeutics. *Drug Discovery Today*. 9, 82-90.

Chobert, J. M., Briand, L., Grinberg, V., Haertle, T. (1995). Impact of esterification on the folding and the susceptibility to peptic proteolysis of beta-lactoglobulin. *Biochimica et Biophysica Acta-Protein Structure and Molecular Enzymology*. 1248, 170-176.

Cocco, R. R., Järvinen, K. M., Han, N., Beyer, K., Sampson, H. A. (2007). Mutational analysis of immunoglobulin E-binding epitopes of β-casein and β-lactogloblulin showed a heterogeneous pattern of clinical amino acids between individual patients and pooled sera. *Clinical and Experimental Allergy*. 37, 831-838.

Collis, A. V., Brouwer, A. P., Martin, A. C. (2003). Analysis of the antigen combining site: correlations between length and sequence composition of the hypervariable loops and the nature of the antigen. *Journal of Molecular Biology*. 325, 337-354.

Cooke, S. K., Sampson, H. A. (1997). Allergenic properties of ovomucoid in man. *The Journal of Immunology*. 159, 2026-2032.

Corzo-Martinez, M., Soria, A. C., Belloque, J., Villamiel, M., Moreno, F. J. (2010). Effect of glycation on the gastrointestinal digestibility and immunoreactivity of bovine beta-lactoglobulin. *International Dairy Journal*. 20, 742-752.

Cunningham, P. T., Elliot, C. E., Lenzo, J. C., Jarnicki, A. G., Larcombe, A. N., Zosky, G. R., Holt, P. G., Thomas, W. R. (2012). Sensitizing and Th2 adjuvant activity of cysteine proteinase allergens. *Int Arch Allergy Immunol*. 158, 347-358.

Dalgalarrondo, M., Dufour, E., Chobert, J. M., Bertrand-Harb, C., Haertlé, T. (1995). Proteolysis of β-lactoglobulin and β-casein by pepsin in ethanolic media. *International Dairy Journal*. 5, 1-14.

Dearman, R. J., Caddick, H., Stone, S., Kenna, J. G., Basketter, D. A., Kimber, I. (2002). Immunogenic properties of rapidly digested food proteins following gavage exposure to mice: a comparison of ovalbumin with a potato acid phosphatase preparation. *Food and Chemical Toxicology*. 40, 625-633.

Defernez, M., Mandalari, G., Mills, E. N. C. (2010). Quantitative assessment of multi-laboratory reproducibility of SDS-PAGE assays: Digestion pattern of beta-casein and beta-lactoglobulin under simulated conditions. *Electrophoresis*. 31, 2838-2848.

Denis, M., Loras-Duclaux, I., Lachaux, A. (2012). Cow's milk protein allergy through human milk. *Archives of Pediatrics and Adolescent Medicine*. 19, 305-312.

Díaz-Perales, A., Blanco, C, Sánchez-Monge, R., Varela, J. Carrillo, T., Salcedo, G. (2003). Analysis of avocado allergen (Prs a 1) IgE-binding peptides generated by simulated gastric fluid digestion. *Journal of Allergy and Clinical Immunology*. 112, 1002-1007.

Dirks, C. G., Pedersen, M. H., Platzer, M. H., Bindslev-Jensen, C., Skov, P. S., Poulsen, L. K. (2005). Does absorption across the buccal mucosa explain early onset of food-induced allergic systemic reactions? *Journal of Allergy and Clinical Immunology*. 115, 1321-1323.

Douliez, J. P., Jegou, S., Pato, C., Larre, C., Molle, D., Marion, D. (2001). Identification of a new form of lipid transfer protein (LTP1) in wheat seeds. *Journal of Agricultural and Food Chemistry*. 49, 1805-1808.

Dunker, A. K., Lawson, J. D., Brown, C. J., Williams, R. M., Romero, P., Oh, J. S., Oldfield, C. J., Campen, A. M., Ratliff, C. M., Hipps, K. W., Ausio, J., Nissen, M. S., Reeves, R., Kang, C., Kissinger, C. R., Bailey, R. W., Griswold, M. D., Chiu, W., Garner, E. C. Obradovic, Z. (2001). *Intrinsically disordered protein. Journal of Molecular Graphics & Modelling*. 19, 26-59.

Dunn, B. M. (2002). Structure and mechanism of the pepsin-like family of aspartic peptidases. *Chemical Reviews*. 102, 4431-4458.

Dupont, D., Mandalari, G., Molle, D., Jardin, J., Le´onill, J., Faulks, R. M., Wickham, M. S. J., Mills, E. N. C., Mackie, A. R. (2010a). Comparative resistance of food proteins to adult and infant in vitro digestion models. *Molecular Nutrition and Food Research*. 54, 767-780.

Dupont, D., Mandalari, G., Mollé, D., Jardin, J., Rolet-Répécaud, O., Duboz, G., Léonil, J., Mills, C. E. N., Mackie, A. R. (2010b). Food processing increases casein resistance to simulated infant digestion. *Molecular Nutrition and Food Research*. 54, 1677-1189.

During, K., Porsch, P., Mahn, A., Brinkmann, O., Gieffers, W. (1999). The non-enzymatic microbicidal activity of lysozymes. *FEBS Letters*. 449, 93-100.

Ehn, B. M. (2004). Process induced changes in IgE binding of the cow's milk allergen beta-lactoglobulin - influence of heat, fermentation and proteolysis. *SIK-Rapport*. 724, 71pp.

Eiwegger, T., Rigby, N., Mondoulet, L, Bernard, H. Krauth, M. T., Boehm, A., Dehlink, E., Valent, P., Wal, J. M., Mills, E. N. C., Szépfalusi, Z. (2006). Gastroduodenal digestion products of the major peanut allergen Ara h 1 retain an allergenic potential. *Clinical & Experimental Allergy*. 36, 1281-1288.

Elsayed, S., Apold, J., Holen, E., Vik, H., Florvaag, E., Dybendal, T. (1991). The structural requirements of epitopes with IgE binding capacity demonstrated by three major allergens from fish, egg and tree pollen. *Scandinavian Journal of Clinical & Laboratory Investigation*. Suppl. 204, 17-31.

Eriksen, E. K., Holm, H., Jensen, E., Aaboe, R., Devold, T. G., Jacobsen, M., Vegarud, G. E. (2010). Different digestion of caprine whey proteins by human and porcine gastrointestinal enzymes. *British Journal of Nutrition*. 104, 374-381.

Ferrua, M. J., Singh, R. P. (2010). Modeling the Fluid Dynamics in a Human Stomach to Gain Insight of Food Digestion. *Journal of Food Science*. 75, R151-R162.

Foltz, M., van Buren, L., Klaffke, W., Duchateau, G. S. M. J. E. (2009). Modeling of the Relationship between Dipeptide Structure and Dipeptide Stability, Permeability, and ACE Inhibitory Activity. *Journal of Food Science*. 74, H243-H251.

Fox, A., Lack, G. (2003). Egg allergy and MMR vaccination. *British Journal of General Practice*. 53, 801-802.

Frémont, S., Kanny, G., Nicolas, P, Moneret-Vautrin, D. A. (1997). Prevalence of lysozyme sensitization in an egg-allergic population. *Allergy*. 52, 224-228.

Friend, D. R. (1991). Colon-specific drug delivery. *Advanced Drug Delivery Reviews*. 7, 149-199.

Fu, T. J., Abbott, U. R., Hatzos, C. (2002). Digestibility of food allergens and nonallergenic proteins in simulated gastric fluid and simulated intestinal fluid- A comparative study. *Journal of Agricultural and Food Chemistry*. 50, 7154-7160.

Fuchs, R. L., Astwood, J. D. (1996). Allergenicity assessment of foods derived from genetically modified plants. *Food Technology*. 50, 83-88.

Furlund, C. B., Kristoffersen, A. B., Devold, T. G., Vegarud, G. E., Jonassen, C. M. (2012). Bovine lactoferrin digested with human gastrointestinal enzymes inhibits replication of human echovirus 5 in cell culture. *Nutrition Research*. 32, 503-513.

Furlund, C. B., Ulleberg, E. K., Devold, T. G., Flengsrud, R., Jacobsen, M., Sekse, C., Holm, H., Vegarud, G. E. (2013). Identification of lactoferrin peptides generated by digestion with human gastrointestinal enzymes. *Journal of Dairy Science*. 96, 75-88.

García-Nebot, M. J., Alegría, A., Barberá, R., Contreras, M. M., Recio, I. (2010). Milk versus caseinophosphopeptides added to fruit beverage: resistance and reléase from simulated gastrointestinal digestión. *Peptides*. 31, 555-561.

García-Redondo, A. B., Roque, F. R., Miguel, M., López-Fandiño, R., Salaices, M. (2010). Vascular effects of egg white-derived peptides in resistance arteries form rats. Structure-activity relatioships. *Journal of the Science of Food and Agriculture*. 90, 1988-1993.

García-Rodríguez, R., Urra, J. M., Feo-Brito, F., Galindo, P. A., Borja, J., Gómez, E., Lara, P., Guerra, F. (2011). Oral rush desensitization to egg: efficacy and safety. *Clinical & Experimental Allergy*. 41, 1289-1296.

Gass, J., Vora, H., Hofmann, A. F., Gray, G. M., Khosla, C. (2007). Enhancement of dietary protein digestion by conjugated bile acids. *Gastroenterology*. 133, 16-23.

Gonzalez-Chavez, S. A., Arevalo-Gallegos, S., Rascon-Cruz, Q. (2009). Lactoferrin: structure, function and applications. *International Journal of Antimicrobial Agents*. 33, 301.e1-8.

Gruber, P., Vieths, S., Wangorsch, A., Nerkamp, J., Hofmann, T. (2004). Maillard reaction and enzymatic browning affect the allergenicity of Pru av 1, the major allergen from cherry (Prunus avium). *Journal of Agricultural and Food Chemistry*. 52, 4002-4007.

Guillou, H., Miranda, G., Pelissier, J. P. (1991). Hydrolysis of β-casein by gastric proteases. comparison of proteolytic action of bovine chymosin and pepsin-a. *International Journal of Peptide Research and Therapeutics*. 37, 494-501.

Hamosh, M., Scanlon, J. W., Ganot, D., Likel, M., Scanlon, K. B., Hamosh, P. (1981). Fat digestion in the newborn - characterization of lipase in gastric aspirates of premature and term infants. *Journal of clinical investigation*. 67, 838-846.

Hanssens, I., Vanceunebroeck, J. C., Pottel, H., Preaux, G., Vancauwelaert, F. (1985). Influence of the protein conformation on the interaction between alpha-lactalbumin and dimyristoyl phosphatidylcholine vesicles. *Biochimica et Biophysica Acta*. 817, 154-164.

Hazebrouck, S., Guillon, B., Drumare, M. F., Paty, E., Wal, J. M., Bernard, H. (2012). Trypsin resistance of the major peanut allergen Ara h 6 and allergenicity of the digestion products are abolished after selective disruption of disulfide bonds. *Molecular Nutrition & Food Research*. 56, 548-557.

Heinzmann, A., Blattmann, S., Spuergin, P., Forster, J., Deichmann, K. A. (1999). The recognition pattern of sequential B cell epitopes of beta-lactoglobulin does not vary with the clinical manifestations of cow's milk allergy. *International Archives of Allergy and Immunology*. 120, 280-286.

Herman, R. A., Woolhiser, M. M., Ladics, G. S., Korjagin, V. A., Schafer, B. W., Storer, N. P., Green, S. B., Kan, L. (2007). Stability of a set of allergens and non-allergens in simulated gastric fluid. *International Journal of Food Sciences and Nutrition*. 58, 125-141.

Herz, U. (2008). Immunological basis and management of food allergy. *Journal of Pediatric Gastroenterology and Nutrition*. 47, Suppl 2, S54-57.

Holen, E., Bolann, B., Elsayed, S. (2001). Novel B and T cell epitopes of chicken ovomucoide (Gal d 1) induce T-cell secretion of IL-6, IL-13 and IFN-gamma. *Clinical & Experimental Allergy*. 31, 952-964.

Holen, E., Elsayed, S. (1996). Specific T cell lines for ovalbumin, ovomucoid, lysozyme and two OA synthetic epitopes, generated from egg allergic patients' PBMC. *Clinical & Experimental Allergy*. 26, 1080-1088.

Holt, C., Sawyer, L. (1993). Caseins as rheomorphic proteins - interpretation of primary and secondary structures of the αs1-caseins, β-caseins and κ-caseins. *Journal of the Chemical Society, Faraday Transactions*. 89, 2683-2692.

Honma, K., Kohno, Y., Saito, K., Shimojo, N., Horiuchi, T., Hayashi, H., Suzuki, N., Hosoya, T., Tsunoo, H., Niimi, H. (1996). Allergenic epitopes of ovalbumin (OVA) in patients with hen's egg allergy: inhibition of basophil histamine release by haptenic ovalbumin peptide. *Clinical & Experimental Immunology*. 103, 446-453.

Horng, J. C., Demarest, S. J., Raleigh, D. P. (2003). pH-dependent stability of the human alpha-lactalbumin molten globule state: Contrasting roles of the 6-120 disulfide and the beta-subdomain at low and neutral pH. *Proteins-structure function and genetics*. 52, 193-202.

Huntington, J. A., Stein, P. E. (2001). Structure and properties of ovalbumin. *Journal of Chromatography B*. 756, 189-198.

Hur, S. J., Lim, B. O., Decker, E. A., McClements, D. J. (2011). *In vitro* human digestion models for food applications. *Food Chemistry*. 125, 1-12.

Iaconelli, A., Fiorentini, L., Bruschi, S., Rossi, F., Mingrone, G., Piva, G. (2008). Absence of allergic reactions to egg white lysozyme additive in Grana Padano cheese. *Journal of the American College of Nutrition*. 27, 326-331.

Ibrahim, H. R., Inazaki, D., Abdou, A., Aoki, T., Kim, M. (2005). Processing of lysozyme at distinct loops by pepsin: A novel action for generating multiple antimicrobial peptide motifs in the newborn stomach. *Biochimica et Biophysica Acta-General Subjects*. 1726, 102-114.

Inglingstad, R. A., Devold, T. G., Eriksen, E. K., Holm, H., Jacobsen, M., Liland, K. H., Rukke, E. O., Vegarud, G.E. (2010). Comparison of the digestion of caseins and whey proteins in equine, bovine, caprine and human milks by human gastrointestinal enzymes. *Dairy Science and Technology*. 90, 549-563.

Jakobson, I., Lindberg, T., Benediktsson, B. (1982). In vito digestion of cow`s milk proteins by duodenal juice from infants with various gastrointestinal disorders. *Journal of Pediatric Gastroenterology and Nutrition*. 1, 183-191.

Järvinen, K. M., Beyer, K., Vila, L., Bardina, L., Mishoe, M., Sampson, H. A. (2007) Specificity of IgE antibodies to sequential epitopes of hen's egg ovomucoid as a marker for persistence of egg allergy. *Allergy*. 62, 758-765.

Järvinen, K. M., Chatchatee, P., Bardina, L., Beyer, K., Sampson, H. A. (2001). IgE and IgG binding epitopes on alpha-lactalbumin and beta-lactoglobulin in cow's milk allergy. *International Archives of Allergy and Immunology*. 126, 111-118.

Jensen-Jarolim, E. (2006). Food safety: In vitro digestion tests are non-predictive for allergenic potential of food in stomach insufficiency. *Immunology Letters*. 102, 118-119.

Jiang, B., Qu, H., Hu, Y., Ni, T., Lin, Z. (2007). Computational analysis of the relationship between allergenicity and digestibility of allergenic proteins in simulated gastric fluid. *BMC bioinformatics*. 8, Article Number: 375.

Jiménez-Saiz, R., Belloque, J., Molina, E., López-Fandiño, R. (2011a). Human immunoglobulin (Ig) E binding to heated and glycated ovalbumin and ovomucoid before and after an in vitro digestión. *Journal of Agricultural and Food Chemistry*. 59, 10044-10051.

Jiménez-Saiz, R., López-Exposito, I., Molina, E., López-Fandiño, R. (2013). IgE-binding and *in vitro* gastrointestinal digestibility of egg allergens in the presence of polysaccharides. *Food Hydrocolloids*. 30, 597-605.

Jiménez-Saiz, R., Martos, G., Carrillo, W., López-Fandiño, R., Molina, E. (2011b). Susceptibility of lysozyme to in-vitro digestión and immunoreactivity of its digests. *Food Chemistry*. 127, 1719-1726.

Jiménez-Saiz, R., Ruiz-Henestrosa, V. M. P. López-Fandino, R., Molina, E. (2012). In vitro digestibility and allergenicity of emulsified hen egg. *Food Research International*. 48, 404-409.

Jolles, P., Jolles, J. (1984). What's new in lysozyme research? *Molecular and Cellular Biochemistry*. 63, 165-189.

Jones, S. M., Pons, L., Roberts, J. L., Scurlock, A. M., Perry, T. T., Kulis, M., Shreffler, W. G., Steele, P., Henry, K. A., Adair, M., Francis, J. M., Durham, S., Vickery, B. P., Zhong, X. P., Burks, A. W. (2009). *Clinical efficacy and immune Immunology*. 124, 292-300.

Joo, K., Kato, Y. (2006). Assessment of allergenic activity of a heat-coagulated ovalbumin after in vivo digestion. *Bioscience, Biotechnology, and Biochemistry*. 70, 591-597.

Kahlert, H., Petersen, A., Becker, W. M., Schlaak, M. (1992). Epitope analysis of the allergen ovalbumin (Gal d II) with monoclonal antibodies and patients' IgE. *Molecular Immunology*. 29, 1191-1201.

Kalantzi, L., Goumas, K., Kalioras, V., Abrahamsson, B., Dressman, J. B., Reppas, C. (2006). Characterization of the human upper gastrointestinal contents under conditions simulating bioavailability/bioequivalence studies. *Pharmaceutical Research*. 23, 165-176.

Keller, J. M., McClellan-Green, P. D., Kucklick, J. R., Keil, D. E., Peden-Adams, M. M. (2006). Effects of organochlorine contaminants on loggerhead sea turtle immunity: Comparison of a correlative field study and *in vitro* exposure experiments. *Environmental Health Perspectives*. 114, 70-76.

Kitabatake, N., Kinekawa, Y. I. (1998). Digestibility of bovine milk whey protein and beta-lactoglobulin in vitro and in vivo. *Journal of Agricultural and Food Chemistry*. 46, 4917-4923.

Koldovsky, O. (1984). Development of human gastrointestinal functions - interaction of changes in diet composition, hormonal maturation, and fetal genetic programming. *Journal of the american college of nutrition*. 3, 131-138.

Kong, F., Singh, R. P. (2010). A Human Gastric Simulator (HGS) to Study Food Digestion in Human Stomach. *Journal of Food Science*. 75, E627-E635.

Kovacs-Nolan, J., Zhang, J. W., Hayakawa, S., Mine, Y. (2000). Immunochemical and structural analysis of pepsin-digested egg white ovomucoid. *Journal of Agricultural and Food Chemistry*. 48, 6261-6266.

Kuwata, H., Yip, T. T., Tomita, M., Hutchens, T. W. (1998). Direct evidence of the generation in human stomach of an antimicrobial peptide domain (lactoferricin) from ingested lactoferrin. *Biochimica et Biophysica Acta-Protein Structure and Molecular Enzymology*. 1429, 129-141.

Leaver, J., Dalgleish, D. G. (1990). The topography of bovine β-casein at an oil/water interface as determined from the kinetics of trypsin-catalysed hydrolysis. *Biochimica et Biophysica Acta/Protein Structure and Molecular Enzymology*. 1041, 217-222.

Ledesma I., Regueras, L., Lapeña, S. (2007). Adverse drug reaction as the form of onset of egg sesitization. *Anales de Pediatría*. 38, 424-425.

Lehrer, S. B., Horner, E., Reese, G. (1996). Why are some proteins allergenic? Implications for biotechnology. *Critical Reviews in Food Science and Nutrition*. 36, 553-564.

Lin, J., Bardina, L., Shreffler, W. G., Andreae, D. A., Ge, Y., Wang, J, Bruni, F. M., Han, Y., Sampson, H. A. (2009). Development of a novel peptide microarray for large scale epitope mapping of food allergens. *Journal of Allergy and Clinical Immunology*. 124, 315-322.

López-Expósito, I., Recio, I. (2006). Antibacterial activity of peptides and folding variants from milk proteins. *International Dairy Journal*. 16, 1294-1305.

López-Expósito, I., Chicon, R., Belloque, J., Recio, I., Alonso, E., López-Fandiño, R. (2008). Changes in the Ovalbumin Proteolysis Profile by High Pressure and Its Effect on IgG and IgE Binding. *Journal of Agricultural and Food Chemistry*. 56, 11809-11816.

López-Expósito, I., López-Fandino, R., Molina, E. (2013). Alergia a alimentos. *Alimentacion, nutricion y salud*. 20, 1-8.

Lyons, P. J., Fricker, L. D. (2011). Carboxypeptidase O is a glycosyl phosphatidyl inositol-anchored intestinal peptidase with acidic amino acid specificity. *Journal of Biological Chemistry*. 286, 39023-39032.

Macierzanka, A., Sancho, A. I., Mills, E. N. C., Rigby, N. M., Mackie, A. R. (2009). Emulsification alters simulated gastrointestinal proteolysis of beta-casein and beta-lactoglobulin. *Soft Matter*. 5, 538-550.

Mackie, A., Macierzanka, A. (2010). Colloidal aspects of protein digestion. *Current Opinion in Colloid and Interface Science*. 15, 102-108.

Mahe, S., Messing, B., Thuillier, F., Tome, D. (1991). Digestion of bovine-milk proteins in patients with a high jejunostomy. *American Journal of Clinical Nutrition*. 54, 534-538.

Mahe, S., Roos, N., Benamouzig, R., Davin, L., Luengo, C., Gagnon, L., Gausserges, N., Rautureau, J., Tome, D. (1996). Gastrojejunal kinetics and the digestion of [N-15]beta-lactoglobulin and casein in humans: The influence of the nature and quantity of the protein. *American Journal of Clinical Nutrition*. 63, 546-552.

Mainville, I., Arcand, Y., Farnworth, E. R. (2005). A dynamic model that simulates the human upper gastrointestinal tract for the study of probiotics. *International Journal of Food Microbiology*. 99, 287-296.

Mandalari, G., Adel-Patient, K., Barkholt, V., Baro, C., Bennett, L., Bublin, M., Gaier, S., Graser, G., Ladics, G. S., Mierzejewska, D., Vassilopoulou, E., Vissers, Y. M., Zuidmeer, L., Rigby, N. M., Salt, L. J., Defernez, M., Mulholland, F., Mackie, A. R., Wickham, M. S. J., Mills, E. N. C. (2009a). In vitro digestibility of beta-casein and beta-lactoglobulin under simulated human gastric and duodenal conditions: A multi-laboratory evaluation. *Regulatory Toxicology and Pharmacology*. 55, 372-381.

Mandalari, G., Mackie, A. M., Rigby, N. M., Wickham, M. S. J., Mills, E. N. C. (2009b). Physiological phosphatidylcholine protects bovine beta-lactoglobulin from simulated gastrointestinal proteolysis. *Molecular Nutrition & Food Research*. 53, S131-S139.

Martínez-Botas, J., Cerecedo, I., Zamora, J., Vlaicu, C., Dieguez, M. C, Gómez-Coronado, D., de Dios, V., Terrados, S., de la Hoz, B. (2013) Mappping of the IgE and IgG4 sequential epitopes of ovomucoid with a peptide microarray immunoassay. *International Archives of Allergy and Immunology*. 161, 11-20.

Martos, G., Contreras, P., Molina, E., López-Fandiño, R. (2010). Egg white ovalbumin digestion mimicking physiological conditions. *Journal of Agricultural and Food Chemistry*. 58, 5640-5648.

Martos, G., López-Expósito, I., Bencharitiwong, R., Berin, M. C., Nowak-Wegrzyn, A. (2011). Mechanisms underlying differential food allergy response to heated egg. *Journal of Allergy and Clinical Immunology.* 127, 990-997.

Martos, G., López-Fandiño, R., Molina, E. (2013). Immunoreactivity of hen egg allergens: influence on *in vitro* gastrointestinal digestión of the presence of other egg white proteins and off egg yolk. *Food Chemistry*. 136, 775-781.

Martos, G., Pineda-Vadillo, C., Miralles, B., Alonso-Lebrero, E., López-Fandiño, R., Molina, E., Belloque, J. (2012). Identification of an IgE reactive peptide in hen egg riboflavin binding protein subjected to simulated gastrointestinal digestion. *Journal of Agricultural and Food Chemistry*. 60, 5215-5220.

Mason S. (1962). Some aspects of gastric function in newborn. *Archives of Disease in Childhood*. 37, 387-391.

Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S. I, Lee, Y. C. (2005). Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. *Analytical Biochemistry*. 339, 69-72.

Matsuda, T., Nakamura, R., Nakashima, I., Hasegawa, Y., Shimokata, K. (1985). Human IgE antibody to the carbohydrate-containing 3rd domain of chicken ovomucoid. *Biochemical and Biophysical Research Communications*. 129, 505-510.

McClements, D. J., Decker, E. A., Park, Y. (2009). Controlling lipid bioavailability through physicochemical and structural approaches. *Critical Reviews in Food Science and Nutrition*. 49, 48-67.

McReynolds, L., O'Malley, B. W., Nisbet, A. D., Fothergill, J. E., Givol, D., Fields, S., Robertson, M., Brownlee, G. G. (1978). Sequence of chicken ovalbumin mRNA. *Nature* 273, 723-728.

Metcalfe, D. D., Astwood, J. D., Townsend, R., Sampson, H. S., Taylor, S. L., Fuchs, R. L. (1996). Assessment of the allergenic potential of foods derived from genetically engineered crop plants. *Critical Reviews in Food Science and Nutrition*. 36, 165-186.

Miguel, M., Recio, I., Gómez-Ruiz, J. A., Ramos, M., López-Fandiño, R. (2004). Angiotensin I-Converting enzyme inhibitory activity of peptides derived from egg white proteins by enzymatic hydrolysis. *Journal of Food Protection.* 67, 1914-1920.

Mills, E. N. C., Madsen, C., Shewry, P. R., Wichers, H. J. (2003). Food allergens of plant origin: their molecular and evolutionary relationships. *Trends in Food Science & Technology*. 14, 145-156.

Mine, Y., Ma, F. P. Lauriau, S. (2004). Antimicrobial peptides released by enzymatic hydrolysis of hen egg white lysozyme. *Journal of Agricultural and Food Chemistry*. 52, 1088-1094.

Mine, Y., Rupa, P. (2003). Fine mapping and structural analysis of immunodominant IgE allergenic epitopes in chicken ovalbumin. *Protein Engineering Design and Selection*. 16, 747-752.

Mine, Y., Yang, M. (2007). Epitope characterization of ovalbumin in BALB/c mice using different entry routes. *BBA-Proteins Proteomics*. 1774, 200-212.

Mine, Y., Yang, M. (2008). Recent advances in the understanding of egg allergens: Basic, industrial, and clinical perspectives. *Journal of Agricultural and Food Chemistry*. 56, 4874-4900.

Mine, Y., Zhang, J. W. (2002a). Comparative studies on antigenicity and allergenicity of native and denatured egg white proteins. *Journal of Agricultural and Food Chemistry*. 50, 2679-2683.

Mine, Y., Zhang, J. W. (2002b). Identification and fine mapping of IgG and IgE epitopes in ovomucoid. *Biochemical and Biophysical Research Communications*. 292, 1070-1074.

Mine, Y., Zhang, M. (2008). Recent Advances in the understanding of egg allergens: basic, industrial, and clinical perspectives. *Journal of Agricultural and Food Chemistry*. 56, 4874-4900.

Minekus, M., Marteau, P., Havenaar, R., huisintveld, J. H. J. A. (1995). multicompartmental dynamic computer-controlled model simulating the stomach and small-intestine. *Atla-Alternatives to Laboratory Animals*. 23, 197-209.

Monaci, L., Tregoat, V., van Hengel, A. J., Anklam, E. (2006). Milk allergens, their characteristics and their detection in food: A review. *European Food Research and Technology*. 223, 149-179.

Moreno, F. J. (2007). Gastrointestinal digestion of food allergens: effect on their allergenicity. *Biomedicine & Pharmacotherapy*. 61, 50-60.

Moreno, F. J., Mellón, F. A., Wickham, M. S. J., Bottrill, A. R., Mills, E. N. C. (2005). Stability of the major allergen Brazil nut 2S albumin (Ber e1) to physiologically relevant *in vitro* gastrointestinal digestion. *FEBS Journal*, 272, 341-352.

Morisawa, Y., Kitamura, A., Ujihara, T., Zushi, N., Kuzume, K., Shimanouchi, Y., Tamura, S., Wakiguchi, H., Saito, H., Matsumoto, K. (2009). Effect of heat treatment and enzymatic digestion on the B cell epitopes of cow's milk proteins. *Clinical and Experimental Allergy*. 39, 918-925.

Mouecoucou, J., Fremont, S., Villaume, C., Sanchez, C., Mejean, L. (2007). Polysaccharides reduce in vitro IgG/IgE-binding of beta-lactoglobulin after hydrolysis. *Food Chemistry*. 104, 1242-1249.

Movérare, R., Elfman, L., Bjornsson, E., Stalenheim, G. (2000). Cytokine production by peripheral blood mononuclear cells following birch-pollen immunotherapy. *Immunology Letters*. 73, 51-56.

Mudgil, P., Torres, M., Millar, T. J. (2006). Adsorption of lysozyme to phospholipid and meibomian lipid monolayer films. *Colloids and Surfaces B-Biointerfaces*. 48, 128-137.

Nisbet, A. D., Saundry, R. H., Moir, A. J. G., Fothergill, L. A., Fothergill, J. E. (1981). The complete amino-acid sequence of hen ovalbumin. *European Journal of Biochemistry*. 115, 335-345.

Niyonsaba, F., Ogawa, H. (2005). Protective roles of the skin against infection: implication of naturally occurring human antimicrobial agents beta-defensins, cathelicidin LL-37 and lysozyme. *Journal of Dermatological Science*. 40, 157-168.

Nowak-Wegrzyn, A., Sampson, H. A. (2006). Adverse reactions to foods. *Medical Clinics of North America*. 90, 97-127.

Nowak-Węgrzyn, A., Sampson, H. A. (2011). Future therapies for food allergies. *Journal of Allergy and Clinical Immunology*. 127, 558-573.

Oliveira, F. M., dos Santos, E. M., Alves, A. C., Campana-Pereira, M. A.); Ramaldes, G. A., Cardoso, V. N., Ruiz-De-Souza, V., Gontijo, C. M. (2007). Digestion, absorption and tissue distribution of ovalbumin and palmitoyl-ovalbumin: Impact on immune responses triggered by orally administered antigens. *Scandinavian Journal of Immunology*. 65, 139-147.

Ongay, S., Boichenko, A., Govorukhina, N., Bischoff, R. (2012). Glycopeptide enrichment and separation for protein glycosylation analysis. *Journal of Separation Science*. 35, 2341-2372.

Pabst, O., Mowat, A. M. (2012). Oral tolerance to food protein. *Mucosal Immunology*. 5, 232-239.

Pal, A., Indireshkumar, K., Schwizer, W., Abrahamsson, B., Fried, M., Brasseur, J. G. (2004). Gastric flow and mixing studied using computer simulation. *Proceedings of the Royal Society B:biological sciences*. 271, 2587-2594.

Pedrosa, C., De Felice, F. G., Trisciuzzi, C., Ferreira, S. T. (2000). Selective neoglycosylation increases the structural stability of vicilin, the 7S storage globulin from pea seeds. *Archives of Biochemistry and Biophysics*. 382, 203-210.

Pellegrini, A., Thomas, U., von Fellenberg, R., Wild, P. (1992). Bactericidal activities of lysozyme and aprotinin against gram-negative and gram-positive bacteria related to their basic character. *Journal of Applied Bacteriology*. 72, 180-187.

Pereira, B., Venter, C., Grundy, J., Clayton, C. B., Arshad, S. H., Dean, T. (2005). Prevalence of sensitization to food allergens, reported adverse reaction to foods, food avoidance, and food hypersensitivity among teenagers. *Journal of Allergy and Clinical Immunology*. 116, 884-892.

Pérez-Calderón, R., Gonzalo-Garijo, M. A., Lamilla-Yerga, A., Mangas-Santos, R., Moreno-Gaston, I. (2007). Recurrent angioedema due to lysozyme allergy. *Journal of Investigational Allergology and Clinical Immunology*. 17, 246-266.

Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., Ferrin, T. E. (2004). UCSF Chimera, a visualization system for exploratory research and analysis. *Journal of Computational Chemistry*. 25, 1605-1612.

Polovic, N., Blanusa, M., Gavrovic-Jankulovic, M., Atanaskovic-Markovic, M., Burazer, L., Jankov, R., Velickovic, T. C. (2007). A matrix effect in pectin-rich fruits hampers digestion of allergen by pepsin in vivo and in vitro. *Clinical and Experimental Allergy*. 37, 764-771.

Polovic, N. D., Pjanovic, R. V., Burazer, L. M., Velickovic, S. J., Jankov, R. M., Velickovic, T. D. C. (2009). Acid-formed pectin gel delays major incomplete kiwi fruit allergen Act c 1 proteolysis in in vitro gastrointestinal digestion. *Journal of the Science of Food and Agriculture*. 89, 8-14.

Polverino de Laureto, P., Frare, E., Gottardo, R., Van Dael H., Fontana. A. (2002). Partly folded status of members of the lysozyme/lactalbumin superfamily: A comparative study by circular dichroism spectroscopy and limited proteolysis. *Protein Science*. 11, 2932-2946.

Pomes, A. (2010). Relevant B cell epitopes in allergic disease. *International Archives of Allergy and Immunology*. 152, 1-11.

Quiros, A., Chichon, R., Recio, I., López-Fandino, R. (2007). The use of high hydrostatic pressure to promote the proteolysis and release of bioactive peptides from ovalbumin. *Food Chemistry*. 104, 1734-1739.

Restani, P., Ballabio, C., Di Lorenzo, C., Tripodi, S., Fiocchi, A. (2009). Molecular aspects of milk allergens and their role in clinical events. *Analytical and Bioanalytical Chemistry*. 395, 47-56.

Restani, P., Gaiaschi, A., Plebani, A., Beretta, B., Cavagni, G., Fiocchi, A., Poiesi, C., Velona, T., Ugazio, A. G., Galli, C. L. (1999). Cross-reactivity between milk proteins from different animal species. *Clinical and Experimental Allergy*. 29, 997-1004.

Roberts, G., Lack, G. (2003). Food allergy and asthma--what is the link? *Paediatric Respiratory Reviews*. 4, 205-212.

Rona, R. J., Keil, T., Summers, C., Gislason, D., Zuidmeer, L., Sodergren, E., Sigurdardottir, S. T., Lindner, T., Goldhahn, K., Dahlstrom, J., McBride, D., Madsen, C. (2007). The prevalence of food allergy: a meta-analysis. *Journal of Allergy and Clinical Immunology*. 120, 638-646.

Rosenberg, D. (2006). Overly precocious food diversification increases risk of allergy: true or false? *Archives de Pediatrie*. 13, 1593-1594.

Ruiter, B., Shreffler, W. G. (2012). Innate immunostimulatory properties of allergens and their relevance to food allergy. *Seminars in Immunopathology*. 34, 617-632.

Rupa, P., Nakamura, S., Mine, Y. (2007). Genetically glycosylated ovomucoid third domain can modulate immunoglobulin E antibody production and cytokine response in

BALB/c mice. *Clinical & Experimental Allergy*. 37, 918-928.

Sackeyfio, A., Senthinathan, A., Kandaswamy, P., Barry, P. W., Shaw, B., Baker, M. (2011). Diagnosis and assessment of food allergy in children and young people: summary of NICE guidance. *British Medical Journal*. 23, 342:d747.

Sato, T., Sasahara, T., Nakamura, Y., Osaki, T., Hasegawa, T., Tadakuma, T., Arata, Y., Kumagai, Y., Katsuki, M., Habu, S. (1994). Naive T-cells can mediate delayed-type hypersensitivity response in T-cell receptor transgenic mice. *European Journal of Immunology*. 24, 1512-1516.

Savage, J. H., Matsui, E. C., Skripak, J. M., Wood, R. A. (2007). The natural history of egg allergy *Journal of Allergy and Clinical Immunology*. 120, 1413-1417.

Savilahti, E. M., Rantanen, V., Lin, J. S., Karinen, S., Saarinen, K. M., Goldis, M., Makela, M. J., Hautaniemi, S., Savilahti, E., Sampson, H. A. (2010). Early recovery from cow's milk allergy is associated with decreasing IgE and increasing IgG4 binding to cow's milk epitopes. *Journal of Allergy and Clinical Immunology*. 125, 1315-1321.

Saw, S., Kale, S. L., Arola, N. (2012). Serine protease inhibitor attenuates ovalbumin induced inflammation in mouse model of allergic airway disease. *PlosOne*. 7, e41107.

Scanff, P., Savalle, B., Miranda, G., Pelissier, J. P., Guilloteau, P., Toullec, R. (1990). *In vivo* gastric digestion of milk-proteins. Effect of technological treatments. *Journal of Agricultural and Food Chemistry*. 38, 1623-1629.

Scanff, P., Yvon, M., Thirouin, S., Pelissier, J. P. (1992). Characterization and kinetics of gastric-emptying of peptides derived from milk-proteins in the preruminant calf. *Journal of Dairy Research*. 59, 437-447.

Scheele, G., Bartelt, D., Bieger, W. (1981). Characterization of human exocrine pancreatic proteins by two-dimensional isoelectric focusing sodium dodecyl sulphate gel electrophoresis. *Gastroenterology*. 80, 461-473.

Schimek, E. M., Zwölfer, B., Briza, P., Jahn-Schmid, B., Vogel, L., Vieths, S., Ebner, C., Bohle, B. (2005). Gastrointestinal digestion of Bet v 1-homologous food allergens destroys their mediator-releasing, but not T cell-activating capacity. *Journal of Allergy and Clinical Immunology*. 116, 1327-1333.

Schmidt, D. G., Meijer, R. J. G. M., Slangen, C. J., Vanberesteijn, E. C. H. (1995). Raising the ph of the pepsin-catalyzed hydrolysis of bovine whey proteins increases the antigenicity of the hydrolysates. *Clinical and Experimental Allergy*. 25, 1007-1017.

Schofield, A. T. (1908). A case of egg poisoning. *Lancet*. 1, 716.

Sélo, I., Clement, G., Bernard, H., Chatel, J., Creminon, C., Peltre, G, Wal, J. (1999) Allergy to bovine beta-lactoglobulin: specificity of human IgE to tryptic peptides. *Clinical & Experimental Allergy*. 29, 1055-1063.

Sharma, S., Kumar, P., Betzel, C., Singh, T. P. (2001). Structure and function of proteins involved in milk allergies. *Journal of Chromatography B*. 756, 183-187.

Shek, L. P., Bardina, L., Castro, R., Sampson, H. A., Beyer, K. (2005). Humoral and cellular responses to cow milk proteins in patients with milk-induced IgE-mediated and non-IgE-mediated disorders. *Allergy*. 60, 912-919.

Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V., Mann M. (2006). In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nature Protocols*. 1, 2856-2860.

Shreffler, W. G., Lencer, D. A., Bardina, L., Sampson, H. A. (2005). IgE and IgG4 epitope mapping by microarray immunoassay reveals the diversity of immune response to the peanut allergen, Ara h 2. *Journal of Allergy and Clinical Immunology*. 116, 893-899.

Sicherer, S. H. (2011). Epidemiology of food allergy. *Journal of Allergy and Clinical Immunology*. 127, 594-602.

Sicherer, S. H., Munoz-Furlong, A., Sampson, H. A. (2004). Prevalence of seafood allergy in the United States determined by a random telephone survey. *Journal of Allergy and Clinical Immunology*. 114, 159-165.

Skripak, J. M., Matsui, E. C., Mudd, K., Wood, R. A. (2007). The natural history of IgE-mediated cow's milk allergy. *Journal of Allergy and Clinical Immunology*.120, 1172-1177.

So, T., Ito, H. O., Hirata, M., Ueda, T., Imoto, T. (1997). Depression of T-cell epitope generation by stabilizing hen lysozyme. *The Journal of Biological Chemistry*. 272, 32136-32140.

So, T.; Ito, H. O., Masato, H., Tadashi, U., Imoto, T. (2001). Contribution of conformational stability of hen lysozyme to induction of type 2 T-helper immune responses. *Immunology*. 104, 259-268.

Sokol, C. L., Barton, G. M., Farr, A. G., Medzhitov, R. A. (2008). Mechanism for the initiation of allergen-induced T helper type 2 responses. *Nature Immunology*. 9, 310-318.

Soto-Mera, M. T., López-Rico, M. R., Filgueira, J. F., Villamil, E., Cidrás, R. (2000). Occupational allergy to papain. *Allergy*. 55, 983-984.

Spratt, P., Nicolella, C., Pyle, D. L. (2005). An engineering model of the human colon. *Food and Bioproducts Processing*. 83, 147-157.

Sreedhara, A., Flengsrud, R., Langsrud, T., Kaul, P., Prakash, V., Vegarud, G. E. (2010). Structural characteristic, pH and thermal stabilities of apo and holo forms of caprine and bovine lactoferrins. *Biometals*. 23, 1159-1170.

Stein, P. E., Leslie, A. G., Finch, J. T., Carrell, R. W. (1991). Crystal structure of uncleaved ovalbumin at 1.95 A resolution. *Journal of Molecular Biology*. 221, 941-959.

Stein, P. E., Leslie, A. G. W., Finch, J. T., Turnell, W. G., McLaughlin, P. J, Carrell, R. W. (1990). Crystal structure of ovalbumin as a model for the reactive centre of serpins. *Nature*. 347, 99-102.

Taheri-Kafrani, A., Gaudin, J. C., Rabesona, H., Nioi, C., Agarwal, D., Drouet, M., Chobert, J. M., Bordbar, A. K., Haertle, T. (2009). Effects of heating and glycation of β-lactoglobulin on its recognition by IgE of sera from cow milk allergy patients. *n*. 11, 4974-4982.

Takagi, K., Teshima, R., Okunuki, H., Itoh, S., Kawasaki, N., Kawanishi, T., Hayakawa, T., Kohno, Y., Urisu, A., Sawada, J. (2005). Kinetic analysis of pepsin digestion of chicken egg white ovomucoid and allergenic potential of pepsin fragments. *International Archives of Allergy and Immunology*. 136, 23-32.

Takagi, K., Teshima, R., Okunuki, H., Sawada, J. I. (2003). Comparative study of *in vitro* digestibility of food proteins and effect of preheating on the digestion. *Biological and Pharmaceutical Bulletin*. 26, 969-973.

Takano, M., Koyama, Y., Nishikawa, H., Murakami, T., Yumoto, R. (2004). Segment-selective absorption of lysozyme in the intestine. *European Journal of Pharmacology*. 502, 149-155.

Tatsumi, E., Yoshimatsu, D., Hirose, M. (1999). Conformational state of disulfide-reduced ovalbumin at acid pH. *Bioscience Biotechnology and Biochemistry*. 63, 1285-1290.

Taylor, S. L., Lehrer, S. B. (1996). Principles and characteristics of food allergens. *Critical Reviews in Food Science and Nutrition*. 36, 91-118.

Teuber, S. S., Sathe, S. K., Peterson, W. R., Roux, K. H. (2002). Characterization of the soluble allergenic proteins of cashew nut (Anacardium occidentale L.). *Journal of Agricultural and Food Chemistry*. 50, 6543-6549.

Thomas, K., Aalbers, M., Bannon, G. A., Bartels, M., Dearman, R. J., Esdaile, D. J., Fu, T. J., Glatt, C. M., Hadfield, N., Hatzos, C., Hefle, S. L., Heylings, J. R., Goodman, R. E., Henry,

B., Herouet, C., Holsapple, M., Ladics, G. S., Landry, T. D., MacIntosh, S. C., Rice, E. A., Privalle, L. S., Steiner, H. Y., Teshima, R., van Ree, R., Woolhiser, M., Zawodny, J. (2004). A multi-laboratory evaluation of a common in vitro pepsin digestion assay protocol used in assessing the safety of novel proteins. *Regulatory Toxicology and Pharmacology*. 39, 87-98.

Thompson, M. J., Eisenberg, D. (1999). Transproteomic evidence of a loop-deletion mechanism for enhancing protein thermostability. *Journal of Molecular Biology*. 290, 595-604.

Troost, F. J., Steijns, J., Saris, W. H. M., Brummer, R. J. M. (2001). Gastric digestion of bovine lactoferrin in vivo in adults. *Journal of Nutrition*. 131, 2101-2104.

Tyssandier, V., Reboul, E., Dumas, J. F., Bougteloup-Demange, C., Armand, M., Marcand, J., Sallas, M., Borel, P. (2003). Processing of vegetable-borne carotenoids in the human stomach and duodenum. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 284, 913-923.

Ulleberg, E. K., Comi, I., Holm, H., Herud, E. B., Jacobsen, M., Vegarud, G. E. (2011). Human gastrointestinal juices intended for use in in vitro digestion models. *Food Digestion*. 2, 52-61.

Untersmayr, E., Jensen-Jarolim, E. (2006). The effect of gastric digestion on food allergy. *Current Opinion in Allergy and Clinical Immunology*. 6, 214-219.

Untersmayr, E., Jensen-Jarolim, E. (2008). The role of protein digestibility and antacids on food allergy outcomes. *Journal of Allergy and Clinical Immunology*. 121, 1301-1308.

Urisu, A., Ebisawa, M., Mukoyama, T., Morikawa, A., Kondo, N. (2011). Japanese Guideline for Food Allergy. *Allergology International*. 60, 221-236.

Van der Beng, B., Chung, E.W., Robinson, C. V., Mateo, P. L., Dobson, C. M. (1999). The oxidative refolding of hen lysozyme and its catalysis by protein disulphide isomerase. *The EMBO Journal*. 18, 4794-4803.

Vance, G. H. S., Lewis, S. A., Grimshaw, K. E. C. Wood, P. J. , Briggs, R. A. Thornton, C. A. Warner, J. O. (2005). Exposure of the fetus and infant to hens' egg ovalbumin via the placenta and breast milk in relation to maternal intake of dietary egg. *Clinical & Experimental Allergy*. 35, 1318-1326.

Vassilopoulou, E., Rigby, N., Moreno, F. J., Zuidmeer, L. Akkerdaas, J., Tassios, I., Papadopoulos, N. G., Saxoni-Papageorgiou, P., van Ree, R., Mills, C. (2006). Effect of *in vivo* gastric and duodenal digestion on the allergenicity of grape lipid transfer protein. *Journal of Allergy and Clinical Immunology*. 118, 473-480.

Venter, C., Pereira, B., Voigt, K., Grundy, J., Clayton, C. B., Higgins, B., Arshad, S. H., Dean, T. (2008). Prevalence and cumulative incidence of food hypersensitivity in the first 3 years of life. *Allergy*. 63, 354-359.

Villas-Boas, M. B., Fernandes, M. A., Zollner, R., Netto, F. M. (2012). Effect of polymerization with transglutaminase on in vitro digestion and antigenicity of β-lactoglobulin. *International Dairy Journal*. 25, 123-131.

Wal, J. M. (1998). An update on allergens. Cow's milk allergens. *Allergy*, 53, 1013-2022.

Walsh, B. J., Hill, D. J., Macoun, P., Cairns, D., Howden, M. E. H. (2005). Detection of four distinct groups of hen egg allergens binding IgE in the sera of children with egg allergy. *Allergologia et immunopathologia*. 33, 183-191.

Wang, J., Lin, J., Bardina, L., Goldis, M., Nowak-Wegrzyn, A., Shreffler, W. G., Sampson, H. A. (2010). Correlation of IgE/IgG4 milk epitopes and affinity of milk-specific IgE antibodies with different phenotypes of clinical milk allergy. *Journal of Allergy and Clinical Immunology*. 125, 695-702.

Watanabe, K., Matsuda, T., Sato, Y. (1981). The secondary structure of ovomucoid and its

domains as studied by circular dichroism. *Biochimica et Biophysica Acta*. 667, 242-250.

Weber, P., Kratzin, H., Brockow, K., Ring, J. Steinhart, H., Paschke, A. (2009). Lysozyme in wine: a risk evaluation for consumers allergic to hen´s egg. *Molecular Nutrition & Food Research.* 53, 1469-1477.

Wills-Karp, M., Nathan, A., Page, K., Karp, C. L. (2010). New insights into innate immune mechanisms underlying allergenicity. *Mucosal Immunology*. 3, 104-110.

Wirnt, R. (1974). In Methods of Enzymatic Analysis. Volume II, 1009-1012, Academic Press Inc. New York, NY.

Wormald, M. R., Dwek, R. A. (1999). Glyoproteins: glycan presentation and protein-fold stability. *Structure with Folding and Design*. 7,155-160.

Wormald, M. R., Wooten, E. W., Bazzo, R., Edge, C. J., Feinstein, A., Rademacher, T. W., Dwek, R. A. (1991). The conformational effects of N-glycosylation on the tailpiece from serum IgM. *European Journal of Biochemistry*. 23, 131-139.

Yamada, K., Urisu, A., Kakami, M., Koyama, H., Tokuda, R., Wada, E., Kondo, Y., Ando, H., Morita, Y., Torii, S. (2000). IgE-binding activity to enzyme-digested ovomucoid distinguishes between patients with contact urticaria to egg with and without overt symptoms on ingestion. *Allergy*. 55, 565-569.

Yousif, A. N., Kan, J. W. (2002). Visualization of chicken ovomucoid in polyacrylamide gels. *Analytical Biochemistry*. 311, 93-97.

Zhang, X. Q., Ge, N., Keiderling, T. A. (2007). Electrostatic and hydrophobic interactions governing the interaction and binding of beta-lactoglobulin to membranes. *Biochemistry*. 46, 5252-5260.

Zhang, J. W., Mine, Y. (1998). Characterization of IgE and IgG epitopes on ovomucoid using egg-white allergic patients' sera. *Biochemical and Biophysical Research Communications*. 253, 124-127.

7. ANNEXES

Annex 1. Specific IgE levels (kU/L) towards milk and β-casein of the sera used in the inhibition ELISA (patients 1 to 6), Western blotting (patients 1 to 4) and microarray assays (patients 7 to 18), shown in section 4.1.

Patient	Age (years)	IgE levels (kU/L)	
		Milk	β-CN
1	2	95.3	91.7
2	6	98.8	90.9
3	7	99.7	94.9
4	5	85.6	62.4
5	1	54.5	48.1
6	1	24.2	32.1
7	1	>100.0	ND
8	1	>100.0	ND
9	0.5	>100.0	ND
10	7	>100.0	ND
11	7	91.3	ND
12	1	30.4	ND
13	2	30.2	ND
14	1	26.4	ND
15	3	19.2	ND
16	4	17.8	ND
17	2	14.2	ND
18	1	5.1	ND

ND: Not determined

Annex 2. Specific IgE levels (kU/L) towards milk and β-lactoglobulin of the sera used in the inhibition ELISA (patients 1 to 6), and microarray assays (patients 7 to 14), shown in section 4.2.

Patient	Age (years)	IgE levels (kU/L)	
		Milk	β-LG
1	6	98.8	62.9
2	5	85.6	34.0
3	10	89.4	31.9
4	1	>100.0	>100.0
5	1	>100.0	>100.0
6	1	27.6	18.9
7	1	26.4	ND
8	2	30.2	ND
9	1	91.3	ND
10	0.5	>100.0	ND
11	1	>100.0	ND
12	1	>100.0	ND
13	0.5	>100.0	ND
14	7	>100.0	ND

ND: Not determined

Annex 3. Specific IgE levels (kU/L) towards egg white, yolk and ovalbumin of the sera used in the inhibition ELISA (patients 1 to 6), Western blotting (patients 7 to 12) and dot blot assays (patients 13 to 22), shown in section 4.3.

Patient	Age (years)	IgE levels (kU/L)		
		Egg white	Egg Yolk	OVA
1	6	40.4	15.8	16.7
2	6	>100	>100	>100
3	7	20	1.67	25.1
4	3	75.9	14.1	65.6
5	4	50.9	16.2	57.7
6	7	41.7	25.7	7.29
7	12	14.7	6.07	2.16
8	3	1.62	0.82	2.89
9	9	11.5	7.25	9.35
10	7	41.7	25.7	7.29
11	11	13.1	6.78	8.33
12	3	>100	90.9	73.7
13	11	54.7	32.9	26
14	7	27.6	20.6	12.3
15	7	41.7	25.7	7.29
16	3	73.2	42.5	46.4
17	12	66	42.9	35.6
18	9	92.7	63.8	76
19	7	>100	>100	70.9
20	3	>100	90.9	73.7
21	6	>100	>100	60.9
22	8	>100	>100	>100

Annex 4. Specific IgE levels (kU/L) towards egg white, yolk and ovomucoid of the sera used in the inhibition ELISA (patients 1-16), Western blotting (patients 1-10), dot blot (patients 1-10) and basophil activation assays (patients 1, 3, 5, 7 and 9), shown in section 4.4.

Patient	Age (years)	IgE levels (kU/L)		
		Egg white	Egg Yolk	OM
1	11	54.7	32.9	37.1
2	7	27.6	20.6	41.1
3	7	41.7	25.7	61.5
4	3	73.2	42.5	62.2
5	12	66	42.9	70.4
6	9	92.7	63.8	77.4
7	7	>100	>100	90.7
8	3	>100	90.9	>100
9	6	>100	>100	>100
10	8	>100	>100	229
11	3	75.9	14.1	46.6
12	7	36.7	5.1	48.3
13	6	>100	>100	87.8
14	6	11.6	4.32	12
15	6	40.4	15.8	49.5
16	4	50.9	42.1	62.1

Annex 5. Specific IgE levels (kU/L) towards egg white, yolk and lysozyme of the sera used in the inhibition ELISA (1-6), Western blotting (patients 1-9), basophil activation (patients 4, 7, 10, 11, 12) and assays of PBMC proliferation assays (patients 13-15), shown in section 4.5.

Patient	Age (years)	IgE levels (kU/L)		
		Egg white	Egg Yolk	LYS
1	12	14.7	6.07	0.35
2	3	1.62	0.82	0.35
3	9	11.5	7.25	0.11
4	7	41.7	25.7	0.12
5	11	13.1	6.78	0.12
6	3	>100	90.9	1.26
7	6	>100	>100	0.59
8	6	>100	>100	2.31
9	6	11.6	4.32	0.71
10	11	54.7	32.9	1.13
11	12	66	42.9	0.12
12	7	>100	>100	13.6
13	10	56.6	44.2	0.85
14	27	5.53	3.08	0.04
15	2	64	13.8	4.3

Annex 6. Peptide sequences identified by RP-HPLC-MS/MS in the gastric (60 min, G60) and gastroduodenal digests of β-CN (60 min of gastric followed by 30 min of duodenal digestion, D30), with simulated and human fluids, shown in section 4.1.

Calculated mass	Sequence	β-CN Fragment	Simulated Fluids		Human Fluids	
			G60	D60	G60	D30
674.324	RELEE	1-5	+	+	+	+
787.408	RELEEL	1-6	+	-	-	-
1283.636	RELEELNVPGE	1-11	+	-	-	-
1624.831	RELEELNVPGEIVE	1-14	+	-	-	-
627.323	LNVPGE	6-11	-	-	+	+
740.407	LNVPGEI	6-12	-	-	-	+
968.518	LNVPGEIVE	6-14	-	-	+	-
627.323	NVPGEI	7-12	-	-	-	+
414.211	PGEI	9-12	-	-	-	+
516.327	KIEK	29 - 32	-	+	-	+
748.288	QQTEDE	39 - 44	-	-	-	+
747.292	EDELQD	42 - 47	-	+	-	-
502.275	LQDK	45 - 48	-	-	-	+
996.539	LQDKIHPF	45 - 52	-	+	-	-
1296.683	LQDKIHPFAQT	45 - 55	+	-	+	-
1511.773	LQDKIHPFAQTQS	45 - 57	+	-	+	-
1624.857	LQDKIHPFAQTQSL	45 - 58	+	-	+	-
883.455	QDKIHPF	46 - 52	-	+	-	-
1183.599	QDKIHPFAQT	46 - 55	+	-	+	-
1398.689	QDKIHPFAQTQS	46 - 57	+	-	+	-
755.397	DKIHPF	47 - 52	-	+	-	-
940.513	KIHPFAQT	48 - 55	-	-	+	-
1155.604	KIHPFAQTQS	48 - 57	-	-	+	-
812.418	IHPFAQT	49 - 55	-	-	+	-
1027.509	IHPFAQTQS	49 - 57	-	-	+	-
646.329	AQTQSL	53 - 58	-	+	-	-
1088.591	SLVYPFPGPI	57 - 66	-	-	-	+
1299.686	SLVYPFPGPIPN	57 - 68	-	-	-	+
1499.802	SLVYPFPGPIPNSL	57 - 70	-	-	+	-
1001.559	LVYPFPGPI	58 - 66	-	-	-	+
1212.654	LVYPFPGPIPN	58 - 68	-	+	-	+
1412.770	LVYPFPGPIPNSL	58 - 70	-	-	+	-
1637.882	LVYPFPGPIPNSLPQ	58 - 72	-	+	-	-
2502.352	LVYPFPGPIPNSLPQNIPPLTQT	58 - 80	+	-	+	-
888.475	VYPFPGPI	59 - 66	-	-	-	+
1099.570	VYPFPGPIPN	59 - 68	-	+	-	+
1299.686	VYPFPGPIPNSL	59 - 70	-	-	+	-
1524.798	VYPFPGPIPNSLPQ	59 - 72	-	+	-	-
2389.268	VYPFPGPIPNSLPQNIPPLTQT	59 - 80	+	-	+	-
529.290	FPGPI	62 - 66	-	-	-	+
1301.734	IPNSLPQNIPPL	66 - 77	-	-	+	-
1307.709	SLPQNIPPLTQT	69 - 80	-	+	-	-
1107.592	PQNIPPLTQT	71 - 80	-	-	+	-

Annex 6. Continuation

Calculated mass	Sequence	β-CN Fragment	Simulated Fluids		Human Fluids	
			G60	D60	G60	D30
882.481	NIPPLTQT	73 - 80	-	-	-	+
2315.260	NIPPLTQTPVVVPPFLQPEVM	73 - 93	-	+	-	-
655.354	PPLTQT	75 - 80	+	-	-	-
994.585	PVVVPPFLQ	81 - 89	-	-	+	-
1220.681	PVVVPPFLQPE	81 - 91	-	-	-	+
1319.749	PVVVPPFLQPEV	81 - 92	+	+	-	+
1450.789	PVVVPPFLQPEVM	81 - 93	+	+	+	-
1507.811	PVVVPPFLQPEVMG	81 - 94	+	-	+	-
1056.531	PPFLQPEVM	85 - 93	+	+	-	-
474.215	PEVM	90 - 93	-	-	+	-
745.433	GVSKVKE	94 - 100	+	-	+	-
2401.265	GVSKVKEAMAPKHKEMPFPKY	94 - 114	-	-	+	-
1709.896	SKVKEAMAPKHKEMP	96 - 110	+	-	-	-
872.479	VKEAMAPK	98 - 105	-	+	-	-
645.316	EAMAPK	100 - 105	-	+	-	+
1673.842	AMAPKHKEMPFPKY	101 - 114	-	-	+	-
1012.516	HKEMPFPK	106 - 113	-	+	-	+
1744.865	HKEMPFPKYPVEPF	106 - 119	-	+	-	-
747.363	EMPFPK	108 - 113	-	+	-	+
1479.711	EMPFPKYPVEPF	108 - 119	-	+	-	-
2125.008	EMPFPKYPVEPFTESQSL	108 - 125	-	+	-	-
603.290	YPVEP	114 - 118	-	-	-	+
750.359	YPVEPF	114 - 119	-	+	-	-
1496.703	YPVEPFTESQSLT	114 - 126	-	+	-	-
440.227	PVEP	115 - 118	-	-	-	+
817.386	PVEPFTE	115 - 121	-	-	+	-
1119.508	PVEPFTESQS	115 - 124	-	-	+	-
794.345	PFTESQS	118 - 124	-	-	+	-
663.308	TESQSL	120 - 125	-	+	-	-
1800.040	LTLTDVENLHLPLPLL	125 - 140	+	+	-	-
790.371	TLTDVEN	126 - 132	-	-	+	+
903.455	TLTDVENL	126 - 133	-	+	-	-
1686.956	TLTDVENLHLPLPLL	126 - 140	+	+	-	-
1902.046	TLTDVENLHLPLPLLQS	126 - 142	-	+	-	-
2088.126	TLTDVENLHLPLPLLQSW	126 - 143	-	+	-	-
1585.908	LTDVENLHLPLPLL	127 - 140	-	+	-	-
576.239	TDVEN	128 - 132	-	-	+	+
1359.740	TDVENLHLPLPL	128 - 139	-	+	-	-
1472.824	TDVENLHLPLPLL	128 - 140	+	+	+	-
1600.882	TDVENLHLPLPLLQ	128 - 141	-	-	+	-
1687.914	TDVENLHLPLPLLQS	128 - 142	+	+	+	-
1371.776	DVENLHLPLPLL	129 - 140	+	+	-	-
1586.867	DVENLHLPLPLLQS	129 - 142	+	+	-	-
1256.749	VENLHLPLPLL	130 - 140	+	+	+	-
1384.808	VENLHLPLPLLQ	130 - 141	+	-	-	-
1471.840	VENLHLPLPLLQS	130 - 142	+	+	-	-
688.427	LHLPLP	133 - 138	-	-	-	+

Annex 6. Continuation

Calculated mass	Sequence	β-CN Fragment	Simulated Fluids		Human Fluids	
			G60	D60	G60	D30
801.511	LHLPLPL	133 - 139	-	+	+	-
914.595	LHLPLPLL	133 - 140	-	-	+	-
1042.654	LHLPLPLLQ	133 - 141	-	-	+	-
1129.686	LHLPLPLLQS	133 - 142	-	-	+	-
365.206	HLP	134 - 136	-	-	-	+
575.343	HLPLP	134 - 138	-	-	-	+
688.427	HLPLPL	134 - 139	-	+	-	-
1016.602	HLPLPLLQS	134 - 142	-	+	-	-
1202.681	HLPLPLLQSW	134 - 143	-	+	-	-
1912.908	QSWMHQPHQPLPPTVM	141 - 156	-	-	+	-
1784.849	SWMHQPHQPLPPTVM	142 - 156	-	-	+	-
1697.817	WMHQPHQPLPPTVM	143 - 156	+	+	+	-
648.280	MHQPH	144 - 148	-	-	-	+
1511.738	MHQPHQPLPPTVM	144 - 156	-	+	-	-
2280.155	MHQPHQPLPPTVMFPPQSVL	144 - 163	-	+	-	-
1013.529	QPHQPLPPT	146 - 154	-	-	-	+
885.471	PHQPLPPT	147 - 154	-	-	-	+
651.359	QPLPPT	149 - 154	-	-	-	+
1350.701	QPLPPTVMFPPQ	149 - 160	-	-	-	+
1437.733	QPLPPTVMFPPQS	149 - 161	-	-	-	+
426.248	LPPT	151 - 154	-	-	-	+
1012.505	PPTVMFPPQ	152 - 160	-	+	-	-
786.428	FPPQSVL	157 - 163	+	+	+	-
639.359	PPQSVL	158 - 163	+	+	-	-
761.428	LSLSQSK	163 - 169	-	+	-	-
520.249	SLSQS	164 - 168	-	-	-	+
648.344	SLSQSK	164 - 169	-	+	-	-
358.258	KVL	169 - 171	-	-	+	-
651.396	VLPVPQ	170 - 175	-	-	-	+
779.491	VLPVPQK	170 - 176	-	+	-	-
1110.644	LPVPQKAVPY	171 - 180	-	-	-	+
371.217	PQK	174 - 176	-	+	-	-
801.438	KAVPYPQ	176 - 182	-	-	-	+
957.540	KAVPYPQR	176 - 183	-	+	-	-
673.344	AVPYPQ	177 - 182	-	-	-	+
829.445	AVPYPQR	177 - 183	-	+	-	-
1484.745	AVPYPQRDMPIQA	177 - 189	-	-	+	-
1314.639	PYPQRDMPIQA	179 - 189	-	-	+	-
474.215	DMPI	184 - 187	-	-	-	+
673.311	DMPIQA	184 - 189	-	+	-	-
933.463	DMPIQAFL	184 - 191	-	+	-	-
462.284	AFLL	189 - 192	+	-	-	-
391.247	FLL	190 - 192	+	-	+	-
1226.691	LLYQEPVLGPV	191 - 201	-	-	-	+
1382.792	LLYQEPVLGPVR	191 - 202	-	+	-	-
2106.224	LLYQEPVLGPVRGPFPIIV	191 - 209	+	+	-	-
1269.708	LYQEPVLGPVR	192 - 202	-	+	-	-

Annex 6. Continuation

Calculated mass	Sequence	β-CN Fragment	Simulated Fluids		Human Fluids	
			G60	D60	G60	D30
1993.140	LYQEPVLGPVRGPFPIIV	192 - 209	+	+	-	-
634.296	YQEPV	193 - 197	-	-	-	+
804.402	YQEPVLG	193 - 199	-	-	+	-
1156.624	YQEPVLGPVR	193 - 202	-	+	-	-
1457.767	YQEPVLGPVRGPF	193 - 205	-	+	+	-
1780.988	YQEPVLGPVRGPFPII	193 - 208	+	+	-	-
1880.056	YQEPVLGPVRGPFPIIV	193 - 209	+	+	+	-
580.358	PVLGPV	196 - 201	-	-	-	+
736.460	PVLGPVR	196 - 202	-	+	-	-
1037.602	PVLGPVRGPF	196 - 205	-	-	+	-
1459.892	PVLGPVRGPFPIIV	196 - 209	+	+	-	-
1150.686	GPVRGPFPIIV	199 - 209	+	-	+	-
1093.665	PVRGPFPIIV	200 - 209	+	-	+	-
685.391	RGPFPI	202 - 207	-	-	-	+
741.443	GPFPIIV	203 - 209	-	+	-	-

Annex 7. Peptide sequences identified by RP-HPLC-MS/MS in the gastric (60 min, G60) and gastroduodenal digests of β-LG (60 min of gastric followed by 30 min of duodenal digestion, D30), with simulated and human fluids, shown in section 4.2.

Calculated mass	Sequence	β-LG Fragment	Simulated Fluids		Human Fluids	
			G60	D60	G60	D30
444.295	LIVT	1-4	-	-	-	+
804.442	LIVTQTM	1-7	+	+	-	-
932.537	LIVTQTMK	1-8	-	+	-	-
1217.669	LIVTQTMKGLD	1-11	+	-	-	-
459.27	IVTQ	2-5	+	+	-	-
819.453	IVTQTMK	2-8	-	+	-	-
876.474	IVTQTMKG	2-9	+	-	-	-
1217.669	IVTQTMKGLDI	2-12	+	-	-	-
892.432	TQTMKGLD	4-11	-	-	+	-
791.385	QTMKGLD	5-11	+	-	+	-
1709.881	TMKGLDIQKVAGTWY	6-20	-	+	-	-
800.476	KGLDIQK	8-14	-	+	-	-
1477.793	KGLDIQKVAGTWY	8-20	-	+	-	-
544.286	GLDIQ	9-13	-	-	-	+
672.381	GLDIQK	9-14	-	+	-	-
715.423	IQKVAGT	12-18	+	-	-	-
901.502	IQKVAGTW	12-19	+	-	+	-
660.36	KVAGTW	14-19	+	+	-	-
532.265	VAGTW	15-19	-	+	-	-
695.328	VAGTWY	15-20	-	+	-	-
1190.622	SLAMAASDISLL	21-32	-	+	-	-
790.39	LAMAASDI	22-29	+	-	-	-
990.506	AMAASDISLL	23-32	+	+	-	-
493.185	MAASD	24-28	+	+	-	-
788.428	AASDISLL	25-32	-	+	-	-
1102.551	AASDISLLDAQ	25-35	-	-	-	+
832.418	ASDISLLD	26-33	-	-	+	+
444.295	ISLL	29-32	+	+	-	-
916.487	ISLLDAQSA	29-37	-	+	-	-
1381.793	ISLLDAQSAPLRV	29-41	+	+	-	-
813.424	LDAQSAPL	32-39	-	+	-	-
1068.593	LDAQSAPLRV	32-41	+	-	-	-
1231.656	LDAQSAPLRVY	32-42	+	-	-	-
700.339	DAQSAPL	33-39	-	-	-	+
856.441	DAQSAPLR	33-40	-	+	-	-
955.509	DAQSAPLRV	33-41	+	-	+	-
769.445	QSAPLRV	35-41	+	-	+	-
436.244	RVY	40-42	-	+	-	-
1587.803	VYVEELKPTPEGDL	41-54	-	-	-	+
1716.846	VYVEELKPTPEGDLE	41-55	-	-	-	+
1943.014	VYVEELKPTPEGDLEIL	41-57	-	+	-	-
2056.098	VYVEELKPTPEGDLEILL	41-58	-	+	-	-
2185.157	VYVEELKPTPEGDLEILLQ	41-59	-	+	-	-

Annex 7. Continuation

Calculated mass	Sequence	β-LG Fragment	Simulated Fluids		Human Fluids	
			G60	D60	G60	D30
538.218	YVEE	42-45	+	+	-	-
779.407	YVEELK	42-47	-	-	+	-
977.507	YVEELKPT	42-49	-	-	-	+
1488.735	YVEELKPTPEGDL	42-54	+	+	+	+
1617.778	YVEELKPTPEGDLE	42-55	+	+	+	+
814.444	VEELKPT	43-49	-	-	-	+
1097.561	VEELKPTPEG	43-52	-	-	-	+
1325.672	VEELKPTPEGDL	43-54	+	+	-	-
1454.714	VEELKPTPEGDLE	43-55	+	+	-	+
1567.798	VEELKPTPEGDLEI	43-56	-	-	-	+
1680.882	VEELKPTPEGDLEIL	43-57	-	+	-	-
1793.967	VEELKPTPEGDLEILL	43-58	-	+	-	-
2050.12	VEELKPTPEGDLEILLQK	43-60	-	+	-	-
1355.646	EELKPTPEGDLE	44-55	-	-	-	+
812.428	ELKPTPE	45-51	+	+	-	-
1226.603	ELKPTPEGDLE	45-55	+	+	+	+
740.407	LKPTPEG	46-52	+	-	-	-
968.518	LKPTPEGDL	46-54	+	+	+	+
1097.561	LKPTPEGDLE	46-55	+	+	-	+
855.434	KPTPEGDL	47-54	+	+	-	+
984.477	KPTPEGDLE	47-55	-	-	-	+
1097.561	KPTPEGDLEI	47-56	+	-	-	+
1895.004	KPTPEGDLEILLQKWE	47-62	-	+	-	-
759.329	TPEGDLE	49-55	-	-	-	+
872.413	TPEGDLEI	49-56	-	-	-	+
658.281	PEGDLE	50-55	-	-	-	+
771.365	PEGDLEI	50-56	-	-	-	+
1228.645	ILLQKWENDE	55-64	-	+	-	-
1658.845	ILLQKWENDECAQK	56-69	-	+	-	-
573.328	LQKW	58-61	-	+	-	-
816.413	LQKWEN	58-63	-	-	+	-
807.286	WENDECA	61-67	+	-	-	-
621.207	ENDECA	62-67	+	-	-	-
2374.257	ENDECAQKKIIAEKTKIPAVF	62-82	-	+	-	-
1637.024	AQKKIIAEKTKIPAV	67-81	-	+	-	-
1784.093	AQKKIIAEKTKIPAVF	67-82	+	+	-	-
372.274	KII	70-72	-	-	-	+
700.449	KIIAEK	70-75	-	+	-	-
444.259	IIAE	71-74	-	+	-	+
572.354	IIAEK	71-75	-	+	-	+
1328.807	IIAEKTKIPAVF	71-82	+	-	-	-
884.533	EKTKIPAV	74-81	+	-	-	-
1031.602	EKTKIPAVF	74-82	+	-	+	-
2974.993	EKTKIPAVFKIDALNENKVLVLDTDY	74-99	-	+	-	-
755.491	KTKIPAV	75-81	+	-	-	-
902.559	KTKIPAVF	75-82	+	-	+	-
528.327	TKIPA	76-80	-	-	-	+

Annex 7. Continuation

Calculated mass	Sequence	β-LG Fragment	Simulated Fluids		Human Fluids	
			G60	D60	G60	D30
774.464	TKIPAVF	76-82	+	+	+	-
545.322	IPAVF	78-82	-	+	-	-
786.501	IPAVFKI	78-84	-	-	+	-
788.444	PAVFKID	79-85	-	-	+	+
1572.888	AVFKIDALNENKVL	80-93	-	-	+	-
948.492	FKIDALNE	82-89	-	+	-	-
445.254	KIDA	83-86	-	-	-	+
801.424	KIDALNE	83-89	+	+	+	-
1043.561	KIDALNENK	83-91	-	+	-	-
1255.714	KIDALNENKVL	83-93	-	+	-	-
1467.866	KIDALNENKVLVL	83-95	+	-	-	-
915.466	IDALNENK	84-91	-	+	-	-
674.287	DALNEN	85-90	-	-	-	+
3033.519	ALNENKVLVLDTDYKKYLLFCMENSA	86-111	-	+	-	-
927.539	NENKVLVL	88-95	+	-	-	-
684.454	NKVLVL	90-95	+	-	-	-
2975.466	KVLVLDTDYKKYLLFCMENSAEPEQ	91-115	-	+	-	-
936.481	VLVLDTDY	92-99	-	+	+	+
1064.576	VLVLDTDYK	92-100	-	+	-	-
2719.313	VLVLDTDYKKYLLFCMENSAEPE	92-114	-	+	-	-
724.328	VLDTDY	94-99	-	+	-	-
852.423	VLDTDYK	94-100	-	+	-	+
980.518	VLDTDYKK	94-101	-	+	-	-
1369.75	VLDTDYKKYLL	94-104	+	-	-	-
1270.681	LDTDYKKYLL	95-104	+	-	-	-
640.271	DTDYK	96-100	-	+	-	-
768.73	DTDYKK	96-101	-	+	-	-
931.429	DTDYKKY	96-102	-	-	+	-
1157.597	DTDYKKYLL	96-104	+	-	+	-
1304.666	DTDYKKYLLV	96-105	+	-	-	-
941.522	DYKKYLL	98-104	-	-	+	-
1652.758	DYKKYLLVCMENS	98-110	-	+	-	-
826.496	YKKYLL	99-104	+	-	-	-
663.432	KKYLL	100-104	+	-	-	-
1446.653	KYLLVCMENSAE	101-112	-	+	-	-
554.311	YLLV	102-105	-	+	-	-
391.247	LLV	103-105	-	+	-	-
1120.435	MENSAEPEQS	107-116	-	-	-	+
1233.519	MENSAEPEQSL	107-117	+	-	-	-
1747.776	ENSAEPEQSLVCQCLV	108-123	-	-	-	+
2004.925	ENSAEPEQSLVCQCLVRT	108-125	-	+	-	-
516.218	NSAEP	109-113	-	+	-	-
860.351	NSAEPEQS	109-116	+	+	-	-
659.277	SAEPEQ	110-115	-	-	-	+
746.309	SAEPEQS	110-116	+	+	-	+
2848.326	EPEQSLACQCLVRTPEVDDEALEKF	112-136	-	+	-	-
908.373	EQSLVCQC	114-121	+	-	-	-
1120.526	EQSLVCQCLV	114-123	-	-	-	+

Annex 7. Continuation

Calculated mass	Sequence	β-LG Fragment	Simulated Fluids		Human Fluids	
			G60	D60	G60	D30
2920.431	QSLVCQCLVRTPEVDDEALEKFD	115-140	-	+	-	-
1760.808	VCQCLVRTPEVDDEAL	118-133	-	+	-	-
1818.813	CQCLVRTPEVDDEALE	119-134	-	+	-	+
1946.908	CQCLVRTPEVDDEALEK	119-135	-	+	-	-
814.419	VRTPEVD	123-129	-	-	-	+
929.446	VRTPEVDD	123-130	+	+	+	+
1058.488	VRTPEVDDE	123-131	+	+	+	+
1129.525	VRTPEVDDEA	123-132	+	+	-	-
1243.609	VRTPEVDDEAL	123-133	+	+	-	-
1499.747	VRTPEVDDEALEK	123-135	-	+	-	-
1889.937	VRTPEVDDEALEKFDK	123-138	-	+	-	-
1272.584	RTPEVDDEALE	124-134	-	-	-	+
559.249	TPEVD	125-129	-	-	-	+
803.319	TPEVDDE	125-131	-	+	-	+
874.356	TPEVDDEA	125-132	-	+	-	-
1116.483	TPEVDDEALE	125-134	-	-	-	+
1244.578	TPEVDDEALEK	125-135	-	+	-	+
1391.646	TPEVDDEALEKF	125-136	-	+	-	-
1634.768	TPEVDDEALEKFDK	125-138	-	+	-	-
1405.625	PEVDDEALEKFD	126-137	+	-	-	-
1690.878	VDDEALEKFDKALKA	128-142	-	+	-	-
575.244	DEALE	130-134	-	-	-	+
721.365	ALEKFD	132-137	-	-	-	+
849.46	ALEKFDK	132-138	-	-	+	+
920.497	ALEKFDKA	132-139	-	-	+	-
1232.713	ALEKFDKALKA	132-142	+	-	+	-
2093.219	ALEKFDKALKALPMHIRL	132-149	+	-	-	-
778.423	LEKFDK	133-138	-	+	-	-
2022.182	LEKFDKALKALPMHIRL	133-149	+	-	-	-
422.217	EKF	134-136	-	-	+	-
665.339	EKFDK	134-138	-	+	-	+
736.376	EKFDKA	134-139	+	+	+	-
849.46	EKFDKAL	134-140	+	-	-	-
977.555	EKFDKALK	134-141	+	-	-	-
1048.592	EKFDKALKA	134-142	+	-	-	-
1526.828	EKFDKALKALPMH	134-146	+	+	-	-
1909.097	EKFDKALKALPMHIRL	134-149	+	-	+	-
536.296	KFDK	135-138	-	+	-	-
919.549	KFDKALKA	135-142	+	-	-	-
1510.87	KFDKALKALPMHI	135-147	+	+	-	-
573.349	DKALK	137-141	-	+	-	-
1504.891	DKALKALPMHIRL	137-149	+	-	-	-
1120.679	KALKALPMHI	138-147	+	-	-	-
742.441	ALKALPM	139-145	-	-	-	+
1261.77	ALKALPMHIRL	139-149	-	-	+	-
1190.732	LKALPMHIRL	140-149	+	-	+	-
949.553	ALPMHIRL	142-149	-	-	+	-
878.516	LPMHIRL	143-149	+	-	+	-

Annex 7. Continuation

Calculated mass	Sequence	β-LG Fragment	Simulated Fluids		Human Fluids	
			G60	D60	G60	D30
424.255	HIR	146-148	-	+	-	-
537.339	HIRL	146-149	-	+	-	-
748.423	IRLSFN	147-152	-	-	+	-
833.44	RLSFNPT	148-154	-	-	-	+
677.339	LSFNPT	149-154	-	-	-	+
564.255	SFNPT	150-154	-	-	-	+
805.397	SFNPTQL	150-156	+	+	+	-
645.297	QLEEQ	155-159	-	-	-	+
757.307	EEQCHI	157-162	+	-	-	-

Annex 8. Peptide sequences identified by RP-HPLC-MS/MS in the gastric (60 min, G60) and gastroduodenal digests of OVA (60 min of gastric followed by 30 min of duodenal digestion, D30), with simulated and human fluids, shown in section 4.3.

Calculated mass	Sequence	OVA Fragment	Simulated Fluids		Human Fluids	
			G60	D60	G60	D30
474.244	TQIN	51-54	-	+	-	+
1103.645	TQINKVVRF	51-59	+	-	+	-
874.539	INKVVRF	53-59	-	+	-	-
1327.798	INKVVRFDKLP	53-63	-	-	-	+
647.412	KVVRF	55-59	+	+	-	-
519.317	VVRF	56-59	+	+	-	-
677.386	RFDKL	58-62	+	-	-	-
1550.773	RFDKLPGFGDSIEA	58-71	-	-	-	+
1176.567	DKLPGFGDSIE	60-70	+	+	+	-
1247.604	DKLPGFGDSIEA	60-71	-	-	-	+
413.264	KLPG	61-64	+	-	-	+
1292.571	LPGFGDSIEAQCG	62-74	-	-	+	-
664.285	AQCGTSV	71-77	-	-	-	+
465.190	CGTSV	73-77	-	-	-	+
754.398	VNVHSSL	77-83	+	+	+	-
655.329	NVHSSL	78-83	+	+	-	-
1719.963	VHSSLRDILNQITKP	79-93	-	-	+	-
689.371	SSLRDI	81-86	-	-	-	+
629.350	RDILN	84-88	-	-	-	+
757.409	RDILNQ	84-89	+	+	+	-
943.534	DILNQITK	85-92	+	+	-	-
813.435	NQITKPN	88-94	-	-	-	+
928.462	NQITKPND	88-95	-	-	-	+
1027.530	NQITKPNDV	88-96	-	-	+	+
1190.593	NQITKPNDVY	88-97	+	+	-	-
1277.625	NQITKPNDVYS	88-98	+	+	+	-
1424.694	NQITKPNDVYSF	88-99	-	-	+	-
686.360	ITKPND	90-95	-	-	-	+
785.429	ITKPNDV	90-96	+	+	+	+
948.492	ITKPNDVY	90-97	+	+	-	-
1035.524	ITKPNDVYS	90-98	+	+	+	-
672.345	TKPNDV	91-96	-	-	-	+
743.313	NDVYSF	94-99	-	-	+	-
829.386	DVYSFSL	95-101	-	-	-	+
792.450	FSLASRL	99-105	+	-	-	-
645.381	SLASRL	100-105	+	+	+	-
445.265	LASR	101-104	+	+	-	-
445.265	ASRL	102-105	-	-	+	-
381.154	YAE	106-108	+	+	-	-
829.361	YAEERY	106-111	+	+	+	-
1152.582	YAEERYPIL	106-114	+	+	+	-
1378.677	YAEERYPILPE	106-116	-	-	-	+
1215.614	AEERYPILPE	107-116	-	-	-	+

Annex 8. Continuation

Calculated mass	Sequence	OVA Fragment	Simulated Fluids		Human Fluids	
			G60	D60	G60	D30
676.355	ERYPI	109-113	-	-	-	+
789.439	ERYPIL	109-114	+	+	+	-
1063.566	KELYRGGLE	122-130	+	-	-	-
1277.677	LYRGGLEPINF	124-134	+	-	-	-
693.345	YRGGLE	125-130	-	-	-	+
1164.593	YRGGLEPINF	125-134	+	+	+	-
740.418	RGGLEPI	126-132	-	-	-	+
854.461	RGGLEPIN	126-133	-	+	-	-
1001.530	RGGLEPINF	126-134	+	+	+	-
845.429	GGLEPINF	127-134	-	-	+	-
1416.689	LEPINFQTAADQA	129-141	-	-	+	-
632.277	QTAADQ	135-140	+	+	+	+
859.415	QTAADQAR	135-142	-	+	-	-
988.458	QTAADQARE	135-143	+	+	+	-
688.314	ADQARE	138-143	-	-	-	+
617.277	DQARE	139-143	-	+	-	-
801.435	ARELINS	141-147	+	+	+	-
987.514	ARELINSW	141-148	-	-	+	-
1645.806	ARELINSWVESQTN	141-154	-	-	-	+
730.398	RELINS	142-147	+	+	-	-
445.254	LINS	144-147	+	+	-	-
519.233	WVES	148-151	-	-	-	+
862.382	WVESQTN	148-154	-	+	-	-
919.404	WVESQTNG	148-155	+	+	+	-
733.325	VESQTNG	149-155	+	+	-	-
400.280	IIR	156-158	-	+	-	-
514.323	IIRN	156-159	-	-	+	-
1224.719	IIRNVLQPSSV	156-166	+	-	-	-
1554.837	IIRNVLQPSSVDSQ	156-169	+	-	-	-
1726.922	IIRNVLQPSSVDSQTA	156-171	+	-	+	-
1857.962	IIRNVLQPSSVDSQTAM	156-172	+	-	-	-
812.451	RNVLQPS	158-164	-	-	-	+
1344.652	NVLQPSSVDSQTA	159-171	-	-	+	-
1475.693	NVLQPSSVDSQTAM	159-172	-	+	-	-
656.350	NVLQPS	159-164	-	-	-	+
542.307	VLQPS	160-164	-	-	-	+
728.407	VLQPSSV	160-166	+	+	-	-
843.434	VLQPSSVD	160-167	-	-	+	-
1058.525	VLQPSSVDSQ	160-169	+	+	-	-
1230.609	VLQPSSVDSQTA	160-171	-	-	+	-
1361.650	VLQPSSVDSQTAM	160-172	+	+	-	-
631.282	QPSSVD	162-167	-	-	+	-
1349.650	SSVDSQTAMVLVN	164-176	-	-	-	+
651.254	DSQTAM	167-172	+	+	-	-
675.432	IVFKGL	178-183	+	-	+	-
861.512	IVFKGLW	178-184	+	-	+	-
830.465	KGLWEKA	181-187	+	-	-	-

Annex 8. Continuation

Calculated mass	Sequence	OVA Fragment	Simulated Fluids		Human Fluids	
			G60	D60	G60	D30
532.265	WEKA	184-187	+	+	-	-
1841.830	WEKAFKDEDTQAMPF	184-198	+	-	-	-
753.318	FKDEDT	188-193	-	-	+	+
952.414	FKDEDTQA	188-195	+	+	+	-
1180.507	FKDEDTQAMP	188-197	+	+	-	-
1327.576	FKDEDTQAMPF	188-198	+	+	+	-
1812.836	FKDEDTQAMPFRVTE	188-202	-	-	+	-
1940.894	FKDEDTQAMPFRVTEQ	188-203	-	-	+	-
878.432	MPFRVTE	196-202	+	-	-	-
1006.491	MPFRVTEQ	196-203	+	-	+	-
1805.881	MPFRVTEQESKPVQM	196-210	+	-	-	-
875.450	PFRVTEQ	197-203	-	-	+	-
1577.787	FRVTEQESKPVQM	198-210	+	+	-	-
503.271	RVTE	199-202	+	+	-	+
631.329	RVTEQ	199-203	+	+	+	+
1171.620	RVTEQESKPV	199-208	-	-	-	+
1299.679	RVTEQESKPVQ	199-209	+	+	-	-
1430.719	RVTEQESKPVQM	199-210	+	+	-	-
1561.760	RVTEQESKPVQMM	199-211	+	+	-	-
686.360	QESKPV	203-208	-	-	-	+
945.459	QESKPVQM	203-210	+	+	+	-
7076.500	QESKPVQMM	203-211	+	+	-	-
558.302	ESKPV	204-208	-	-	-	+
686.360	ESKPVQ	204-209	+	+	-	-
817.401	ESKPVQM	204-210	+	+	+	-
948.441	ESKPVQMM	204-211	+	+	-	-
723.363	MYQIGL	211-216	-	-	+	-
592.322	YQIGL	212-216	+	+	+	-
748.423	GLFRVAS	215-221	-	-	-	+
709.358	FRVASM	217-222	+	+	-	-
562.290	RVASM	218-222	+	+	+	-
693.301	VASMASE	219-225	-	-	+	-
1193.615	VASMASEKMKI	219-229	-	-	+	-
692.353	ASEKMK	223-228	+	+	+	-
805.437	ASEKMKI	223-229	-	-	+	-
1160.648	ASEKMKILEL	223-232	+	-	+	-
631.409	KMKIL	226-230	-	-	+	-
745.441	MKILEL	227-232	-	+	+	-
486.306	ILEL	229-232	-	+	-	-
709.311	PFASGTM	233-239	+	+	+	-
927.383	PFASGTMSM	233-241	+	+	+	-
797.454	LVLLPDE	242-248	+	+	+	-
896.522	LVLLPDEV	242-249	-	-	-	+
684.370	VLLPDE	243-248	+	+	-	-
1040.576	VLLPDEVSGL	243-252	+	+	+	-
828.423	LPDEVSGL	245-252	+	+	-	-
631.318	VSGLEQ	249-254	-	-	-	+

Annex 8. Continuation

Calculated mass	Sequence	OVA Fragment	Simulated Fluids		Human Fluids	
			G60	D60	G60	D30
574.297	ESIIN	256-260	-	-	-	+
721.365	ESIINF	256-261	+	+	+	-
618.323	EKLTE	262-266	+	+	+	+
804.402	EKLTEW	262-267	+	+	-	-
1193.557	EKLTEWTSSN	262-271	-	-	+	-
823.335	TEWTSSN	265-271	-	-	+	-
593.245	WTSSN	267-271	+	+	+	-
1307.727	MEERKIKVYL	273-282	+	-	-	-
1063.603	EERKIKVY	274-281	+	-	-	-
1176.687	EERKIKVYL	274-282	+	-	-	-
672.429	ERKIK	275-279	+	+	-	-
934.560	ERKIKVY	275-281	+	-	-	-
1047.644	ERKIKVYL	275-282	+	-	+	-
387.285	KIK	277-279	-	+	+	-
649.417	KIKVY	277-281	-	+	-	-
762.501	KIKVYL	277-282	-	-	+	-
521.322	IKVY	278-281	-	+	-	-
634.406	IKVYL	278-282	-	+	-	-
887.560	IKVYLPR	278-284	-	-	+	-
774.425	LPRMKM	282-287	+	-	-	-
661.341	PRMKM	283-287	+	-	-	-
666.272	MKMEE	285-289	-	-	-	+
794.367	MKMEEK	285-290	-	-	-	+
826.390	KMEEKY	286-291	-	+	-	-
698.295	MEEKY	287-291	-	+	-	-
567.254	EEKY	288-291	-	+	-	-
836.377	MAMGITDV	298-305	+	+	-	-
983.446	MAMGITDVF	298-306	+	-	-	-
852.405	AMGITDVF	299-306	-	+	-	-
781.368	MGITDVF	300-306	+	+	-	-
650.328	GITDVF	301-306	+	+	-	-
823.408	VFSSSANL	305-312	+	+	-	-
725.323	FSSSADL	306-312	+	+	+	-
1079.510	SSSANLSGISSA	307-318	+	+	-	-
736.324	SGISSAES	313-320	-	-	-	+
849.408	SGISSAESL	313-321	+	+	-	-
803.439	ESLKISQ	319-325	+	+	-	-
1460.774	ESLKISQAVHAAHA	319-332	+	-	-	-
1945.986	ESLKISQAVHAAHAEINE	319-336	+	-	-	-
3243.612	ESLKISQA……VVGSAEA	319-350	+	-	-	-
587.365	LKISQ	321-325	-	-	+	-
459.306	LKIS	321-324	-	-	-	+
1729.911	LKISQAVHAAHAEINE	321-336	-	-	+	-
781.445	KISQAVH	322-328	-	+	-	-
1131.615	KISQAVHAAHA	322-332	+	-	+	-
1616.827	KISQAVHAAHAEINE	322-336	+	-	+	-
2030.030	KISQAVHAAHAEINEAGRE	322-340	+	-	-	-

Annex 8. Continuation

Calculated mass	Sequence	OVA Fragment	Simulated Fluids		Human Fluids	
			G60	D60	G60	D30
655.350	ISQAVH	323-328	-	+	-	-
675.346	AVHAAHA	326-332	+	+	+	-
1160.558	AVHAAHAEINE	326-336	+	+	+	-
1231.595	AVHAAHAEINEA	326-337	-	-	+	-
1573.760	AVHAAHAEINEAGRE	326-340	+	-	-	-
1986.987	AVHAAHAEINEAGREVVGSA	326-345	+	-	-	-
853.393	AAHAEINE	329-336	+	+	-	-
1266.596	AAHAEINEAGRE	329-340	+	+	-	-
1679.823	AAHAEINEAGREVVGSA	329-345	+	+	-	-
916.425	EINEAGRE	333-340	+	+	-	-
374.180	INE	334-336	-	-	-	+
844.441	AGREVVGSA	337-345	+	+	+	-
973.483	AGREVVGSAE	337-346	+	-	+	-
1315.637	AGREVVGSAEAGVD	337-350	+	+	-	-
902.435	VVGSAEAGVD	341-350	+	+	-	-
631.282	AEAGVDA	345-351	-	-	+	-
1628.743	VDAASVSEEFRADHP	349-363	-	-	+	-
691.303	AASVSEE	351-357	+	+	+	-
1750.832	ASVSEEFRADHPFLF	352-366	-	-	+	-
1130.551	EFRADHPFL	357-365	+	-	-	-
741.356	FRADHP	358-363	-	-	-	+
888.425	FRADHPF	358-364	-	+	-	-
1001.509	FRADHPFL	358-365	+	-	+	-
854.440	RADHPFL	359-365	+	+	-	-
438.187	ADHP	360-363	-	-	-	+
681.418	IKHIAT	368-373	+	+	-	-
795.461	IKHIATN	368-374	-	-	+	-
866.498	IKHIATNA	368-375	-	-	+	-
1730.893	HIATNAVLFFGRCVSP	370-385	-	+	-	+

Annex 9. Peptide sequences identified by RP-HPLC-MS/MS in the gastric (60 min, G60) and gastroduodenal digests (60 min of gastric followed by 30 min of duodenal digestion, D30) of OM and dOM with simulated fluids, shown in section 4.4.

Calculated mass	Sequence	OM Fragment	OM		dOM	
			G60	D60	G60	D30
778.328	AEVDCSR	1-7	-	+	-	+
925.326	AEVDCSRF	1-8	-	-	+	-
1207.529	AEVDCSRFPNA	1-11	+	-	-	-
1423.604	AEVDCSRFPNATD	1-13	-	-	+	-
1980.885	AEVDCSRFPNATDKEGKD	1-18	-	+	-	+
603.313	RFPNA	7-11	-	-	-	+
1432.720	FPNATDKEGKDVL	8-20	+	-	-	-
902.449	DKEGKDVL	13-20	-	-	-	-
575.19	KEGKD	14-18	-	-	-	-
787.444	KEGKDVL	14-20	-	-	+	+
1728.901	GKDVLVCNKDLRPICG	16-31	-	-	+	-
917.333	KDVLVCNK	17-24	-	-	-	-
674.379	VLVCNK	19-24	-	+	-	+
789.405	VLVCNKD	19-25	+	+	+	+
1644.833	VLVCNKDLRPICGTD	19-33	+	-	-	-
1701.854	VLVCNKDLRPICGTDG	19-34	+	-	+	-
1800.923	VLVCNKDLRPICGTDGV	19-35	-	-	+	-
1901.970	VLVCNKDLRPICGTDGVT	19-36	+	-	+	-
2065.034	VLVCNKDLRPICGTDGVTY	19-37	+	+	+	+
2166.081	VLVCNKDLRPICGTDGVTYT	19-38	+	-	+	-
1432.680	VCNKDLRPICGTD	21-33	+	-	+	-
1489.702	VCNKDLRPICGTDG	21-34	+	-	+	-
1689.818	VCNKDLRPICGTDGVT	21-36	+	+	+	+
1852.881	VCNKDLRPICGTDGVTY	21-37	+	+	+	+
1953.929	VCNKDLRPICGTDGVTYT	21-38	+	-	-	-
2399.092	VCNKDLRPICGTDGVTYTNDCL	21-42	+	-	-	-
741.244	NKDLRP	23-28	-	-	-	-
740.454	KDLRPI	24-29	+	-	+	-
988.465	DLRPICGTD	25-33	-	-	-	+
1245.602	DLRPICGTDGVT	25-36	-	+	-	+
1408.666	DLRPICGTDGVTY	25-37	-	+	-	+
1509.713	DLRPICGTDGVTYT	25-38	-	+	-	-
930.459	LRPICGTDG	26-34	-	-	+	-
1130.575	LRPICGTDGVT	26-36	+	+	+	+
1293.639	LRPICGTDGVTY	26-37	-	-	+	-
1040.485	VTYTNDCLL	35-43	+	-	-	-
511.104	YTND	37-40	-	-	-	-
727.285	YTNDCL	37-42	+	+	+	+
1014.415	YTNDCLLCA	37-45	+	+	+	+
564.221	TNDCL	38-42	-	+	-	+
851.352	TNDCLLCA	38-45	-	+	-	-
1432.637	CLLCAYSIEFGTN	41-53	-	-	-	+
1103.53	CAYSIEFGTN	44-53	-	-	-	-

Annex 9. Continuation

Calculated mass	Sequence	OM Fragment	OM		dOM	
			G60	D60	G60	D30
1766.811	AYSIEFGTNISKEHDG	45-60	-	+	-	-
510.233	YSIE	46-49	+	+	+	+
657.301	YSIEF	46-50	+	+	+	+
679.264	EFGTNI	49-54	-	-	-	-
437.113	FGTN	50-53	-	-	-	-
1144.518	ISKEHDGECK	54-63	-	+	-	+
1701.77	ISKEHDGECKETVPM	54-68	-	-	-	+
816.307	EHDGECK	57-63	-	-	-	+
679.262	DGECKE	59-64	-	-	+	-
575.262	ETVPM	64-68	-	-	-	+
1830.813	SYANTTSEDGKVMVLCN	72-88	+	-	-	-
734.345	TSEDGKV	77-83	-	-	+	-
865.346	TSEDGKVM	77-84	-	-	-	-
548.263	DGKVM	80-84	-	+	-	+
647.322	DGKVMV	80-85	-	-	-	-
760.415	DGKVMVL	80-86	+	-	+	-
961.520	KVMVLCNR	82-89	-	+	-	-
805.394	MVLCNRA	84-90	+	-	+	-
603.316	VLCNR	85-89	-	+	-	+
674.353	VLCNRA	85-90	-	-	+	+
1507.691	VLCNRAFNPVCGTD	85-98	+	-	-	-
1564.713	VLCNRAFNPVCGTDG	85-99	+	-	+	-
1764.829	VLCNRAFNPVCGTDGVT	85-101	+	-	-	-
2042.919	VLCNRAFNPVCGTDGVTYD	85-103	+	-	+	-
933.381	LCNRAFNP	86-93	-	-	-	-
1465.644	LCNRAFNPVCGTDG	86-99	-	-	+	-
462.201	CNRA	87-90	-	-	-	+
609.269	CNRAF	87-91	-	+	-	-
1552.676	CNRAFNPVCGTDGVT	87-101	+	-	+	-
1830.767	CNRAFNPVCGTDGVTYD	87-103	-	-	+	-
1613.714	RAFNPVCGTDGVTYD	89-103	+	-	+	-
922.385	AFNPVCGTD	90-98	-	-	-	+
979.407	AFNPVCGTDG	90-99	-	-	-	+
1342.586	AFNPVCGTDGVTY	90-102	-	+	-	+
1457.613	AFNPVCGTDGVTYD	90-103	-	+	-	+
851.348	FNPVCGTD	91-98	-	-	-	+
908.37	FNPVCGTDG	91-99	-	-	-	+
1386.576	FNPVCGTDGVTYD	91-103	-	+	-	+
704.280	NPVCGTD	92-98	-	+	-	+
961.417	NPVCGTDGVT	92-101	-	+	-	+
1124.481	NPVCGTDGVTY	92-102	-	+	-	+
1239.508	NPVCGTDGVTYD	92-103	-	+	-	+
1125.465	PVCGTDGVTYD	93-103	+	+	+	+
553.238	GVTYD	99-103	-	+	-	-
1068.480	VTYDNECLL	100-108	-	+	-	+
1764.781	TYDNECLLCAHKVEQ	101-115	-	-	+	-
868.364	YDNECLL	102-108	-	-	-	+

Annex 9. Continuation

Calculated mass	Sequence	OM Fragment	OM		dOM	
			G60	D60	G60	D30
590.273	NECLL	104-108	-	+	-	+
764.320	NECLLCA	104-110	-	+	-	+
1513.702	NECLLCAHKVEQGA	104-117	+	-	+	-
1600.734	NECLLCAHKVEQGAS	104-118	+	-	+	-
1699.802	NECLLCAHKVEQGASV	104-119	+	-	-	-
1357.648	CLLCAHKVEQGAS	106-118	+	-	+	-
1141.555	LCAHKVEQGAS	108-118	+	+	+	+
870.402	CAHKVEQG	109-116	-	+	-	+
1028.471	CAHKVEQGAS	109-118	-	+	-	+
1127.539	CAHKVEQGASV	109-119	-	+	-	-
1242.566	CAHKVEQGASVD	109-120	-	+	-	+
1526.762	CAHKVEQGASVDKR	109-122	-	+	-	+
925.462	AHKVEQGAS	110-118	-	+	-	+
1267.652	AHKVEQGASVDK	110-121	-	-	+	-
1423.753	AHKVEQGASVDKR	110-122	-	-	-	+
767.338	HKVEQGA	111-117	-	-	-	-
854.425	HKVEQGAS	111-118	+	+	+	+
953.409	HKVEQGASV	111-119	-	-	-	-
717.366	KVEQGAS	112-118	-	+	-	+
1215.657	KVEQGASVDKR	112-122	-	+	-	+
988.494	EQGASVDKR	114-122	-	+	-	-
1613.733	EQGASVDKRHDGGCR	114-128	-	+	-	-
1097.521	GASVDKRHDGG	116-126	-	-	-	+
926.457	ASVDKRHD	117-124	-	-	-	+
1040.529	ASVDKRHDGG	117-126	-	-	-	-
1740.869	SVDKRHDGGCRKELAA	118-133	+	-	+	-
516.302	VDKR	119-122	-	+	-	-
1398.679	VDKRHDGGCRKE	119-130	-	-	+	-
1511.763	VDKRHDGGCRKEL	119-131	-	-	+	-
1582.800	VDKRHDGGCRKELA	119-132	+	-	+	-
1653.837	VDKRHDGGCRKELAA	119-133	+	-	+	-
1554.769	DKRHDGGCRKELAA	120-133	+	-	-	-
1901.884	RHDGGCRKELAAVSVDCS	122-139	-	+	-	-
643.250	HDGGCR	123-128	-	+	-	+
771.345	HDGGCRK	123-129	-	+	-	+
900.387	HDGGCRKE	123-130	-	+	-	-
1013.471	HDGGCRKEL	123-131	-	+	-	+
1155.545	HDGGCRKELAA	123-133	-	+	-	-
1493.697	GGCRKELAAVSVDCS	125-139	+	-	-	-
718.248	CRKELA	127-132	-	-	-	-
402.211	ELAA	130-133	-	+	-	+
3162.358	AVSVDCSEYP……PLCGSDNKTY	133-161	-	+	-	-
1741.718	VSVDCSEYPKPDCTAE	134-149	+	+	+	+
2222.982	VSVDCSEYPKPDCTAEDRPL	134-153	-	+	-	+
2827.210	VSVDCSEYPKPDCTAEDRPLCGSDNK	134-159	-	+	-	-
1555.617	VDCSEYPKPDCTAE	136-149	+	+	+	+
2036.882	VDCSEYPKPDCTAEDRPL	136-153	-	+	-	+

Annex 9. Continuation

Calculated mass	Sequence	OM Fragment	OM		dOM	
			G60	D60	G60	D30
1341.522	CSEYPKPDCTAE	138-149	+	+	+	+
1822.787	CSEYPKPDCTAEDRPL	138-153	-	+	-	-
622.296	SEYPK	139-143	-	-	-	+
859.375	PKPDCTAE	142-149	+	-	-	-
1340.639	PKPDCTAEDRPL	142-153	-	+	-	-
1018.439	DCTAEDRPL	145-153	-	-	-	+
1380.529	DCTAEDRPLCGSD	145-157	-	-	-	+
800.403	TAEDRPL	147-153	-	-	-	+
1061.445	AEDRPLCGSD	148-157	-	+	-	-
1103.503	DRPLCGSDNK	150-159	-	+	-	+
1367.614	DRPLCGSDNKTY	150-161	-	+	-	+
1515.645	LCGSDNKTYGNKCN	153-166	+	-	+	-
622.238	CGSDNK	154-159	-	-	-	+
886.349	CGSDNKTY	154-161	-	+	-	+
1040.427	NKTYGNKCN	158-166	-	-	-	-
681.290	GNKCNF	162-167	-	+	-	-
1105.507	CNAVVESNGTL	168-178	-	+	-	+
1319.639	CNAVVESNGTLTL	168-180	-	+	-	+
718.350	VESNGTL	172-178	-	+	-	+
819.402	VESNGTLT	172-179	-	-	-	-
932.481	VESNGTLTL	172-180	+	+	+	+
603.302	TLSHF	179-183	-	+	-	+
788.418	TLSHFGK	179-185	-	-	-	+
891.427	TLSHFGKC	179-186	+	+	+	+
790.380	LSHFGKC	180-186	+	-	+	+
677.296	SHFGKC	181-186	+	+	+	-